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THE METABOLISM OF γ-HYDROXYGLUTAMIC-α-C^{14} ACID IN THE INTACT RAT[1]

BY LÉO BENOITON AND L. P. BOUTHILLIER

Abstract

Synthetic γ-hydroxyglutamic-α-C^{14} acid was found to be extensively catabolized in the intact rat and to give rise to both labelled glutamic and aspartic acids. The results obtained are in agreement with a previously proposed theory that γ-hydroxyglutamic acid may be converted to glutamic and aspartic acids by two distinct metabolic routes. The fact that only a small amount of radiocarbon was found in the tissue hydroxyproline suggests that the conversion of γ-hydroxyglutamic acid to hydroxyproline, if it does occur, is of minor importance.

Introduction

Until very recently, γ-hydroxyglutamic acid had never been detected in nature, nor had it ever been synthesized. Last year, Virtanen and Hietala isolated this amino acid from the green parts of *Phlox decussata* (23), and they found decarboxylase activity for it in *Escherichia coli* (24). The decarboxylation product was α-hydroxy-γ-aminobutyric acid. At about the same time, γ-hydroxyglutamic acid was synthesized in our laboratory (1). However, nothing is as yet known concerning the metabolism of γ-hydroxyglutamic acid in mammalian tissue.

Two groups of workers have proposed γ-hydroxyglutamic acid as a possible intermediate in the degradation of hydroxyproline. Taggart and Krakaur incubated L-hydroxyproline in the presence of washed insoluble particles of homogenized rabbit kidney and they succeeded in isolating, from the incubation medium, a 2,4-dinitrophenylosazone corresponding to that of γ-hydroxyglutamic acid semialdehyde (21). This led them to propose that, in analogy with the degradation of proline to glutamic acid, the γ-hydroxyglutamic acid semialdehyde would then be oxidized to γ-hydroxyglutamic acid. The work of Taggart and Krakaur has been confirmed by Lang and Mayer who claimed to have crystallized and purified the 2,4-dinitrophenylosazone of γ-hydroxyglutamic acid semialdehyde (14).

More recently, Gianetto and Bouthillier have studied the degradation of hydroxyproline with the aid of radioactive carbon (8). These authors injected DL-hydroxyproline-2-C^{14} into three rats and found that, among the amino

[1]*Manuscript received February 28, 1956.*
Contribution from the Department of Biochemistry, Faculty of Medicine, University of Montreal, Montreal, Quebec.

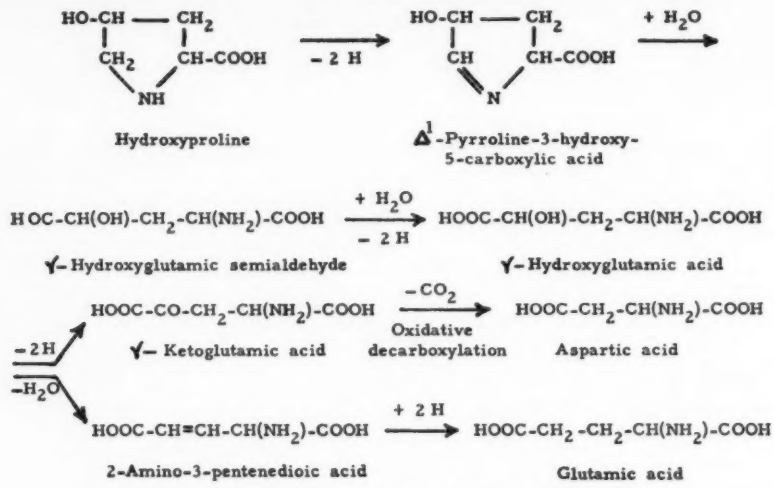

FIG. 1. Proposed scheme for the degradation of hydroxyproline.

acids isolated from the tissue proteins, glutamic and aspartic acids were particularly rich in isotope. After studying the distribution of the C^{14} in these acids, they arrived at the conclusion that these two amino acids were very probably formed by two distinct routes. In the light of their results, they presented a scheme, illustrated in Fig. 1, in which γ-hydroxyglutamic acid is proposed as a common intermediate in the formation of glutamic and aspartic acids from hydroxyproline.

In this paper, we describe the *in vivo* experiments which we have performed with radioactive γ-hydroxyglutamic acid.

Experimental

Synthesis of γ-Hydroxyglutamic-α-C^{14} Acid

This compound was synthesized by a method previously described (1), except that ethyl acetamidocyanoacetate was employed instead of diethyl acetamidomalonate.* The scheme for the synthesis appears in Fig. 2. Ethyl acetamidocyanoacetate-α-C^{14} (0.117 gm., 0.5 mc.) diluted with nonradioactive product (0.733 gm.) was condensed with ethyl α-acetoxy-β-chloropropionate (1.3 gm.) in anhydrous alcohol (10 ml.) containing sodium (0.13 gm.). After removing the sodium chloride and alcohol, the mixture was refluxed four hours in 20% hydrochloric acid (15 ml.). The evaporated hydrolyzate was then placed on a 4.5 × 50 cm. column of Dowex 50 (acid form), the amino acids were eluted with 1.2 *N* hydrochloric acid, and 15 ml. fractions were collected. After the solvent was removed under vacuum, the amino acid was obtained by precipitation with alcohol from water (4 ml.) adjusted to pH 2.7 with tributylamine. Yield: 0.30 gm.; m.p. 170° C.

**This change has increased yields from about 35 to 55% on the basis of 0.25 molar quantities.*

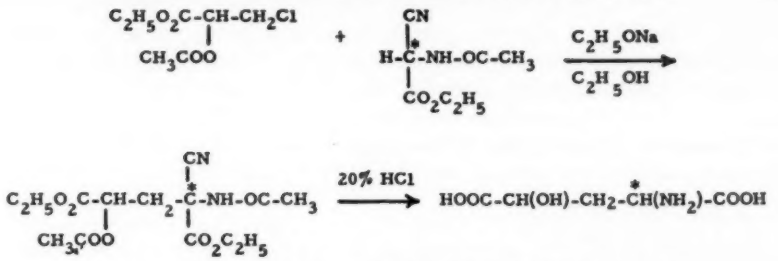

FIG. 2. Scheme for the synthesis of γ-hydroxyglutamic-α-C¹⁴ acid.

Paper chromatograms in two solvents (phenol : water, 4 : 1; *n*-butanol: acetic acid : water, 15 : 3 : 7) gave only one radioactive peak which coincided with the ninhydrin spot. The specific activity of the γ-hydroxyglutamic-α-C¹⁴ acid monohydrate (diastereomeric mixture) was 1.6×10^5 counts per minute per milligram.

Biological Experiments

Doses of the labelled substance were given by intraperitoneal injection to three male rats of Wistar strain, weighing 50 gm. each. Rat 1 received a 1 ml. solution (pH 7.4) of 10 mgm. of γ-hydroxyglutamic-α-C¹⁴ acid, while Rats 2 and 3 each received 20 mgm. Rats 1 and 2 were placed in a glass metabolism cage, the urine was collected, and the respiratory carbon dioxide was absorbed by a 10% solution of sodium hydroxide and precipitated as barium carbonate. The two animals were sacrificed with ether after 24 and 12 hr. and their entire tissues were minced in a Waring blendor in the presence of 10% tricholoroacetic acid. Rat 3 was sacrificed after 12 hr. and the liver only was minced in trichloroacetic acid. Several amino acids were then isolated from these crude proteins.

Tissue Amino Acids

The proteins were hydrolyzed in 20% hydrochloric acid, and the tyrosine was removed by isoelectric precipitation at pH 6.0. Glutamic and aspartic acids were precipitated as their barium salts, and the free acids, obtained by acidification with sulphuric acid, were then separated by chromatography on a 4.5 × 100 cm. column of Dowex 50 (acid form) with 1.2 *N* hydrochloric acid as the eluting agent (17). This system provides a complete separation for these two amino acids. The glutamic acid was crystallized as its hydrochloride, while the aspartic acid, after further purification by copper salt formation, was crystallized as the free acid. For Rat 3, both acids were diluted, immediately before barium salt formation, by one part amino acid as calculated from the amino acid composition of rat liver proteins (2, 7). The specific activity of all dicarboxylic amino acid samples remained the same after one recrystallization.

Samples of glutamic and aspartic acids were decarboxylated by means of ninhydrin according to the method of Van Slyke *et al.* (22) and the carbon dioxide was collected as barium carbonate.

The prolines (Rat 1 only) were obtained together as the reineckates which were decomposed by treatment with pyridine (10). They were then separated by column chromatography as in the case of the dicarboxylic amino acids. After removal of the hydrochloric acid, proline was crystallized as the cadmium chloride complex (11), and the hydroxyproline was obtained in the free state from water–alcohol.

Radioactivity Measurements

Known quantities of the substances to be examined were uniformly spread on stainless steel cups (4.5 cm.^{2}) and their radioactivity was measured with a D-47 micromil window counter (Nuclear-Chicago).

Chromatography of Urine

The techniques employed were the same as those previously reported (13). The solvents used were *n*-butanol : acetic acid : water (15 : 3 : 7) and phenol saturated with borate buffer of pH 9.3 (15).

Results and Discussion

The rates of C^{14} excretion in the respiratory carbon dioxide of Rats 1 and 2 are represented in Fig. 3. In both cases, the maximum excretion occurred in the second hour period. The amount of radiocarbon appearing in the carbon dioxide was 47% of the dose for Rat 1 (24 hr.) and 37% for Rat 2 (12 hr.). The rate of excretion follows a pattern comparable to that reported for other amino acids such as glycine (3, 9), histidine (3, 6), and glutamic acid (4). In the latter case, 35% of a dose of DL-glutamic-γ-$C^{14}OOH$ acid was excreted as carbon dioxide in eight hours, the maximum excretion having occurred during the first hour.

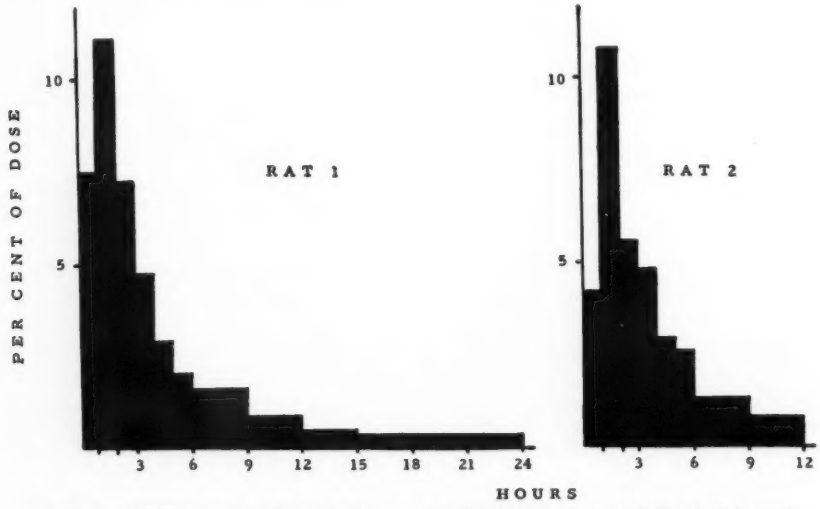

FIG. 3. Isotope excretion in respiratory carbon dioxide as per cent of C^{14} administered.

The urine of Rats 1 and 2 contained 43 and 46% of the injected radioactivity. Paper chromatography indicated that essentially all the urinary radioactivity was due to unchanged γ-hydroxyglutamic acid.

The radioactivity of isolated tissue amino acids is given in Table I. The glutamic and aspartic acids were found to be the most radioactive. Treatment of the glutamic acid with ninhydrin indicated that 13 to 20% of the radiocarbon present in the molecule was located in the α-carboxyl group (Table II). The labelling of this carbon is a consequence of the utilization of carbon dioxide in the formation of oxalacetate from pyruvate. This same reaction may also be responsible for the incorporation of isotope into the other carbons of the glutamic acid molecule, but only to a much smaller extent (12). Since over 80% of the radiocarbon content of the tissue glutamic acid was located in positions other than the α-carboxyl group, it is obvious that the major part of this isotope came directly from γ-hydroxyglutamic-α-C^{14} acid or perhaps indirectly by some metabolic pathway other than the carboxylation reaction.

The aspartic acid, by similar ninhydrin decarboxylation, was found to contain 27 to 65% of its total radioactivity in the carboxyl groups combined (Table II). In this case also, carboxylation reactions cannot account in any way for the presence of 35 to 73% of the radiocarbon in atoms 2 and 3 of aspartic acid, therefore this isotope must also have come from γ-hydroxyglutamic acid by some other pathway or pathways.

In so far as the distribution of the radioactivity in the glutamic and aspartic acid molecules is concerned, our results indicate that the two dicarboxylic

TABLE I

RADIOACTIVITY OF TISSUE AMINO ACIDS

Rat No.	Duration of experiment, hr.	Dose injected, c./min.	Tissue analyzed	Specific activity, c./min./mM.			
				Glutamic acid	Aspartic acid	Proline	Hydroxy-proline
1	24	1.5×10^6	Carcass	4040	2640	1120	740
2	12	2.9×10^6	Carcass	12,100	7470	—	—
3*	12	3.0×10^6	Liver	16,500	11,800	—	—

**Corrected for a onefold dilution.*

TABLE II

DISTRIBUTION OF C^{14} IN GLUTAMIC AND ASPARTIC ACIDS

Rat No.	Glutamic acid			Aspartic acid		
	Specific activity, c./min./mM.	Specific activity of ninhydrin-liberated CO_2 (alpha-COOH), c./min./mM.	% C^{14} in alpha-COOH	Specific activity, c./min./mM.	Specific activity of ninhydrin-liberated CO_2 (both COOH), c./min./mM.	% C^{14} in both COOH
1	4040	810	20.0	2640	860	65.0
2	12,100	2050	17.0	7470	1830	49.0
3	8250	1090	13.2	5900	790	26.8

acids may have been formed from γ-hydroxyglutamic acid by two distinct pathways. Thus our results are consistent with the previously proposed theory that the degradation of hydroxyproline to glutamic and aspartic acids proceeds by way of γ-hydroxyglutamic acid as a common intermediate. However, we must exercise extreme prudence before concluding without a doubt that in our experiments two separate catabolic pathways were actually involved. We cannot disregard the possibility that the incorporation of the isotope into one of the dicarboxylic amino acids could have resulted from their interconversion, and that the labelled γ-hydroxyglutamic acid was in fact catabolized directly to only one of the two dicarboxylic amino acids.

For the first possibility, γ-hydroxyglutamic acid could have been catabolized directly to glutamic acid, which in turn could have been converted to aspartic acid. Since the γ-hydroxyglutamic acid was labelled in carbon 2, it is logical to assume that the isotope in the resulting glutamic acid should be present as well in carbon 2. However, the conversion of glutamic-2-C^{14} acid to aspartic acid, by way of the ketoglutarate–succinate–oxalacetate system, can only result in molecules of aspartic acid being labelled in carbons 1 and 4. To explain the labelling of the aspartic acid in carbons 2 and 3, the conversion would have to proceed through a reversal of the tricarboxylic acid cycle, that is, by way of ketoglutarate–oxalosuccinate–citrate–oxalacetate. Our results indicate that a substantial amount (35 to 73%, Table II) of the radioactivity contained in the aspartic acid was located in positions 2 and 3. This would require that this reversal of the tricarboxylic acid cycle be responsible to the same extent as its normal operation for the conversion of the glutamic acid to aspartic acid. If one considers that the equilibrium constant for the reaction catalyzed by the condensing enzyme is 10^6 in favor of citrate formation (18), it is very improbable that this reverse reaction could account for so large an incorporation of isotope in aspartate. Therefore the interconversion of glutamate–aspartate cannot be responsible for the extensive labelling of aspartic acid, and consequently, most of its radioactivity must have come from γ-hydroxyglutamic-α-C^{14} acid by a direct pathway.

For the second possibility, γ-hydroxyglutamic acid could have been degraded directly to aspartic which in turn could have been converted to glutamic acid by way of oxalacetate–citrate–ketoglutarate. However, the fact that in all three experiments the specific activity of the aspartic acid was less than that of the glutamic acid would indicate that this reaction system was not the major route by which the isotope was incorporated into the glutamic acid. Even though our knowledge of the biological dilution and relative turnover rates of the two dicarboxylic acids is limited, it still seems improbable that the isotope present in the glutamic acid originated in the aspartic acid. Most of the radioactivity in the glutamic acid must have come from the γ-hydroxyglutamic-α-C^{14} acid by a direct pathway.

Therefore, our results indicate strongly that γ-hydroxyglutamic acid is catabolized to aspartic and to glutamic acids by two distinct pathways. It is hoped that further work will provide us with conclusive evidence.

Now let us consider the radioactivity data (Table I) concerning the prolines isolated from the tissue proteins. The isotope contained in the proline very probably originated from its known precursor glutamic acid (5, 16), while that in the hydroxyproline could have come from the proline (19, 20). What is noteworthy is the fact that the results offer no indication that γ-hydroxyglutamic acid could be a direct precursor of hydroxyproline.

One may note that the γ-hydroxyglutamic acid which we used in the experiments did not consist of only one isomer. The synthesis was such that the product obtained was very probably a mixture of the four possible isomers. Before any metabolic study could be undertaken, we had to establish whether or not such a mixture could exert any harmful effect on the animals. With this view in mind we administered massive doses of 250 mgm. of normal γ-hydroxyglutamic acid to several 100 gm. rats. The product was well tolerated by all animals and caused no apparent ill effects. There is therefore no reason to believe that the results obtained in our metabolic experiments represent anything other than the true physiological picture. The fact remains that in these experiments, about half of the amino acid administered was utilized by the animals; at least one of the isomers must have been converted to glutamic and to aspartic acids.

Acknowledgment

The authors are indebted to the 'Fondation Joseph Rhéaume', Montreal, Quebec, for supporting this research.

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THE ACTION OF FACTOR I ON CERTAIN INVERTEBRATE ORGANS[1]

By Ernst Florey

Abstract

Factor I, prepared from beef brain, has been applied to a number of invertebrate organs and physiological preparations. It was found to antagonize the effect of acetylcholine in some preparations while it imitated it in others. The discussion of the results of this investigation as well as of previous findings leads to the assumption that factor I may be classified with the many pharmacological agents which affect cholinergic mechanisms and that it is likely to be a transmitter substance of inhibitory neurons.

Introduction

It has previously been reported that the mammalian central nervous system contains a principle, factor I, which imitates the actions of peripheral inhibitory neurons in crustaceans, e.g. it slows or stops the heart, inhibits the discharge of stretch receptor neurons, and blocks neuromuscular transmission (6). It has also been observed that this factor blocks the action of acetylcholine on the stretch receptors as well as on the crayfish intestine (6, 7). In mammals, solutions containing factor I cause block of synaptic transmission in certain autonomic ganglia (9, 10) and in the monosynaptic extensor reflex arcs (9, 11). These solutions inhibit spontaneous activity of the rabbit ileum and, in this organ as well as in the ileum of the guinea pig, they block the action of the acetylcholine (provided that the solution applied is slightly acid) (5). It seems of particular interest that factor I preparations also exhibit excitatory actions. They cause facilitation and stimulation in polysynaptic spinal reflex arcs and stimulate the hypoglossal nucleus of the cat's brain (11).

The chemical nature of factor I is not yet known but this is being studied. Meanwhile it appeared to be of interest to collect more information about the actions of this factor in non-vertebrate physiological preparations. It was hoped that such studies might lead to some explanation of the ability of the extracts to cause inhibition in some preparations and excitation in others. It was also hoped that the observations made might, in the light of our present knowledge of invertebrate pharmacology, provide some clues to the chemical nature of factor I.

Methods

Factor I was prepared in the following way:* Fresh beef brain, 1 kgm., was boiled for 10 min. in water. The mixture was homogenized and dialyzed in cellophane tubing against six volumes of water. The dialyzate was

[1]*Manuscript received January 10, 1956.*

Contribution from the Lerner Marine Laboratory, Bimini, Bahamas, B.W.I., and the Donner Laboratory for Experimental Neurochemistry, Montreal Neurological Institute, McGill University, Montreal, Canada. This work was aided by grants to Dr. K. A. C. Elliott from the Banting Research Foundation and the Ciba Company, Ltd., Montreal.

**A more detailed description of the method of purification and assay of factor I will be published elsewhere (Elliott and Florey (4)).*

concentrated and lead acetate was added until no further precipitate occurred. The precipitate was removed by centrifuging, the solution was gassed with hydrogen sulphide, filtered, and aerated. Perchloric acid was added in amount equivalent to the potassium content of solution and the precipitate of potassium chlorate was filtered off. The filtrate was concentrated to 15 ml., chilled, and further precipitate was filtered off in the cold. This solution was then twice extracted with 25 ml. of ether and afterwards thoroughly aerated.

The dry weight of this solution was found to be 232 mgm./ml. Of this 0.96 mgm. was found to be K, 87.4 mgm. Na, and 37.5 mgm. Cl. Since the K and Na must be present as salts (partly chloride, acetate, and perchlorate) only a very small proportion of the solid matter of this preparation could be organic material.

The activity of this preparation was assayed on the crayfish stretch receptor. It was found to contain 2400 crayfish units* per ml. Since the activity of the original brain was found to be 120 crayfish units per gm. fresh weight, 1 ml. of the final solution of factor I contained the activity of 20 gm. of fresh brain.

For convenient reference the activity of factor I will be expressed in *brain equivalents* (B.E. (9)); 1 B.E. is the amount of activity found in 1 gm. of fresh beef brain.

The solution was carried by air to the island of Bimini, B.W.I., and was stored there at −5° C. Fresh dilutions were made up daily from this stock solution which, because of its high salt content, did not freeze at the storage temperature. Since the stock solution had a pH of 3.5 the first dilution (usually 1 : 5) was neutralized by addition of 2 *N* NaOH. The first dilution of 1 : 5 was made with distilled water, since the salt content of the original solution was about 20% and since a salt concentration of about 3.7% was required to make the solution isotonic with sea water. All further dilutions were made with fresh, filtered sea water.

Drugs

All drug solutions were prepared in fresh filtered sea water shortly before each experiment. The dilutions referred to in this paper are the final dilutions of samples in contact with the respective physiological preparations.

Physiological Preparations

(1) Longitudinal Muscles of Sea Cucumbers

Sea cucumbers of the species *Actinopyga agassizi* Selenka were freshly caught before each experiment. They were cut open longitudinally and eviscerated. A 2–5 cm. length of the white longitudinal muscles, depending on the state of contraction, was dissected off the cuticula and transferred to a dish of clean sea water. One end of the muscle strip was then tied to the bottom of a bathing vessel which held 10 ml. The other end of the muscle was connected with the writing lever of a slow-moving kymograph. A load

*See footnote on page 669.

of 1–2 gm. pulled on the muscle. The bathing fluid (fresh, filtered sea water) was constantly aerated and mixed by a stream of air bubbles. Samples to be tested were injected into the bath.

(*2*) *Spine Movements of Sea Urchins*

Freshly caught animals of the species *Centrechinus antillarum* Phillipi were opened, eviscerated, and rinsed out with fresh sea water. Segments of the shell were cut out and fixed in a clamp. All the large spines were broken off near their bases except one which was left intact. A thread was attached to this spine, at a distance of about 5 cm. from its base, and connected with a very light writing lever. Solutions to be tested were sprayed with a syringe over the inner surface of the shell. They could be removed by spraying with fresh aerated sea water.

(*3*) *Lobster Heart*

The abdomens of spiny lobsters (*Palinurus argus*) were removed and the animals were allowed to bleed. The dorsal part of the carapace was then removed, the pericardium was carefully opened and the frontal and lateral arteries were tied off. One thread of this tie was later connected with the writing lever of the kymograph. A cannula was inserted into the abdominal artery. The sternal artery was tied off. The heart was then carefully cut loose from its connections and, by way of the cannula, it was perfused with aerated sea water from a Mariotte bottle at a pressure of 50 cm. sea water. The hearts released the perfusion fluid through their ostia at a rate of about 20 ml. per minute. Samples to be tested were injected into the rubber tubing that connected the cannula with the sea water container.

(*4*) *Crab Claws*

Chelipeds of several species of crabs (mainly *Callinectes exasperatus* Gerstaecker, *Portunus spinimanus* Latreille, and *Gecarcinus* sp. were amputated at the basis of the ischiopodite. The exoskeleton of the meropodite was removed from the ventral aspect and the flexor muscles of the carpopodite were dissected off. The nerve bundle was split lengthwise and the efferent axones were lifted up one after the other to the tips of thin platinum electrodes. The axones were stimulated at a rate of approximately 20 impulses per second using an induction coil. This procedure of changing the axones was continued until either the "fast" or the "slow" motor neuron of the adductor muscle of the claw was found.

The tip of the propodite was cut off and a two way cannula was inserted. One of its inlets was connected with a reservoir of filtered and aerated sea water; through the other, solutions of factor I could be injected with the aid of a 1 ml. tuberculin syringe.

The tip of the dactylopodite was connected with the writing lever of the kymograph. An appropriate weight was used to keep the claw open when not stimulated.

(5) *Cephalopod Hearts*

Freshly caught specimens of *Sepiotheuthis sepioidea* d. Orb. and *Octopus vulgaris* L. were decerebrated and opened from the back. The cephalic artery and the right auricle as well as the visceral arteries were tied off. A cannula was inserted into the left auricle and tied in. The heart was then cut loose from its connections. The stump of the cephalic artery was connected to the writing lever of the kymograph and the heart was mounted in the upright position by fixing the cannula with an appropriate clamp. The heart was filled and perfused with sea water from a Mariotte bottle. The ligature on one of the small visceral arteries was cut off so that the heart could pump out fluid. The rate of flow was adjusted to about 20 ml./min. Samples to be tested were injected into the rubber tubing which connected the cannula with the sea water reservoir. The sea water used was constantly aerated.

(6) *Cephalopod Rectum*

With most of the animals used for heart preparations the rectum was also dissected. This was done by carefully cutting it loose from its strong connections with the ink sack. Most of the preparations were kept in the refrigerator for a few hours before use but some were used immediately after dissection. They were mounted in a bathing vessel which held 10 ml. of fluid, and the "spontaneous" contractions were recorded on a slow-moving kymograph. The sea water used as bathing fluid was constantly aerated and mixed by a stream of air bubbles in the bath.

(7) *Longitudinal Muscle of Leech*

Dorsal muscle strips of *Hirudo medicinalis* L. were dissected and set up in the usual way for routine assays of acetylcholine. The experiments in which leech muscles were used were done in Montreal. The saline solution used had the following composition: NaCl 0.66%, KCl 0.01%, $CaCl_2$ 0.02%, and glucose 0.1%. Samples to be tested were injected into the bathing fluid.

Results

(1) The longitudinal muscles of sea cucumbers are known to be very sensitive to acetylcholine (1, 14). Uneserinized mucles of *Actinopyga* were found to respond to acetylcholine 10^{-11}. Factor I solutions, even in concentrations as high as 1 B.E./ml., did not block or diminish the action of subsequently applied acetylcholine. Sufficiently concentrated solutions of factor I actually caused contraction and seemed to potentiate the effect of a low concentration of acetylcholine if this was applied without intercurrent rinsing (Fig. 1). It was observed that application of a very low concentration of acetylcholine potentiates the effect of subsequently applied acetylcholine. It seemed likely, therefore, that the effect of the factor I solution on the sea cucumber muscle was due to the presence of a trace of acetylcholine in the factor I preparation. This conclusion was confirmed by finding that the effect was strongly potentiated by eserine (Fig. 2). A dilution of the factor I

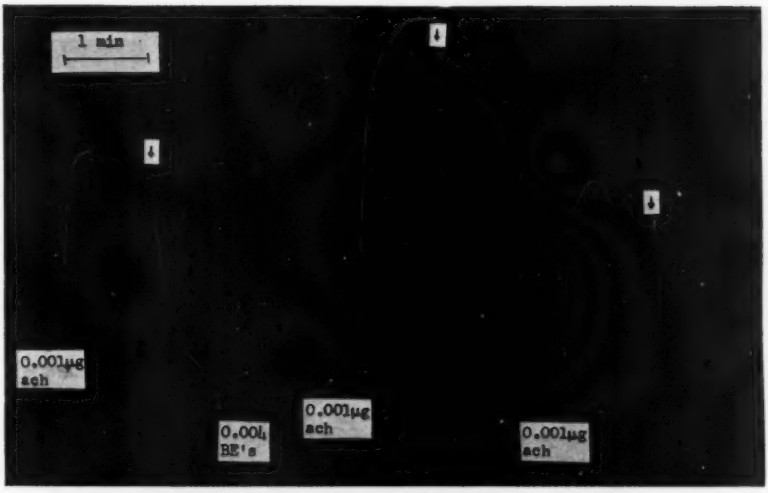

FIG. 1. Isolated longitudinal muscle of a sea cucumber (*Actinopyga agassizi*). Kymograph record of contractions induced by acetylcholine (ach) and factor I (expressed in brain equivalents, B.E.). The amounts indicated had been added to the 10 ml. bath. At the arrows the solutions were exchanged for fresh sea water.

solution which contained 0.002 B.E./ml. behaved like 0.00002 µgm./ml. of acetylcholine, whereas it would have contained about 0.004 µgm./ml. if all the acetylcholine that was originally present in the beef brain were contained in the original factor I preparation. The factor I preparation thus contained about 0.5% of the original acetylcholine content of the tissue. Results of experiments on leech muscles confirm this estimate (see paragraph 7).

(2) Reflexes of sea urchins, particularly of *Centrostephanus*, *Sphaerechinus*, *Arbacia*, *Dorocidaris*, and *Echinus* have been extensively studied and described by v. Uexkuell (15). In the present study it has been observed with specimens of *Centrechinus antillarum* Phillipi that there is a distinct response to illumination. In the undisturbed animal there is no direct response to a sudden increase in light but a sudden decrease of illumination, or a passing shadow, provokes a rapid movement of the spines of the darkened area. If the spines are in the "locked" position (which occurs as a reaction to traction) they usually show motion as soon as there is an increase in light. These reflexes remain intact in isolated segments of the shell. When 1 ml. of sea water containing 0.1 B.E. of factor I was sprayed onto the inside of the shell a transient excitation occurred in a few cases but, in all preparations, a complete flaccid paralysis of the spine muscles occurred after a brief delay. Reflex movements, normally elicited by light or touch, ceased. This paralysis was reversible (Fig. 3).

(3) The lobster heart was found to be extremely sensitive to factor I. A total amount of 0.00025 B.E. injected into the perfusion cannula was sufficient to cause complete diastolic arrest. In contrast to earlier observations on the

FIG. 2. Isolated longitudinal muscle of a sea cucumber (*Actinopyga agassizi*). Kymograph record of contractions induced by acetylcholine (ach) and factor I (expressed in B.E. per ml.). After addition of eserine (es) the effect of factor I is potentiated, which indicates the presence of acetylcholine in the factor I preparation. At the arrows the muscle was washed.

crayfish heart (6), there were no normal heart beats during the inhibition. During the recovery period the size of the heart beats built up gradually until it reached the original amplitude.

The presence of an inhibitory factor in the crustacean nervous system was indicated in previous reports (6, 8). It was of interest to compare its action with that of factor I prepared from beef brain. The effect of 1 ml. of an extract prepared by boiling 100 mgm. of lobster nerve cord in 10 ml. of sea water was nearly identical with the effect of 1 ml. of sea water containing 0.002 B.E. of factor I (Fig. 4). The effect of an extract of lobster leg nerves was even more powerful. The extract from 1 mgm. of leg nerve in 1 ml. of sea water contained enough inhibitory activity to stop the heart completely. A comparison of several assays showed that leg nerves contain four to five times more inhibitory activity per unit of fresh weight than the ventral nerve cord. As can be seen from Fig. 4, there were no normal heart beats during the action of the inhibitory principle from the lobster nervous system.

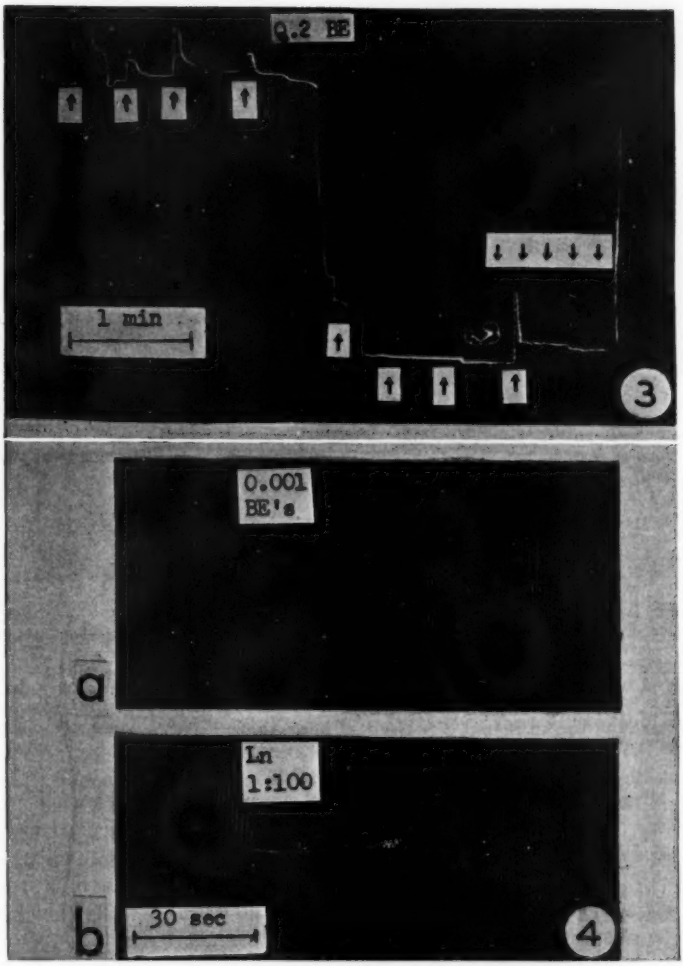

FIG. 3. Kymograph record of spine movements of a sea urchin (*Centrochinus antillarum*). The arrows pointing up indicate a two second flash of light. Arrows pointing down indicate spraying with fresh sea water. Spraying with 0.2 B.E. of factor I effects a complete loss of tone. There is no response to light.

FIG. 4. Isolated, perfused heart of the lobster (*Palinurus argus*). Kymograph record of the effect of the heart beat of (*a*) 0.001 B.E. of factor I and (*b*) 1 ml. of an extract of lobster leg nerve 1 : 100.

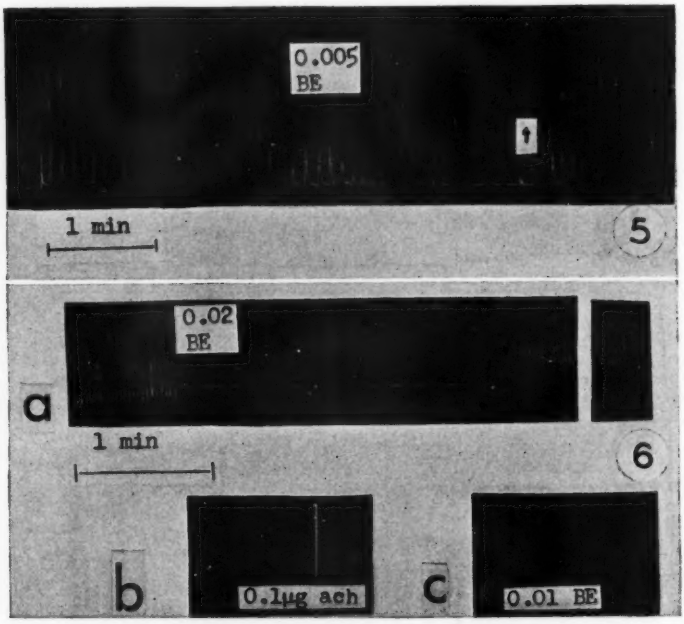

FIG. 5. Isolated claw of the crab *Portunus spinimanus*. Kymograph record of the movements of the dactylopodite during maximal stimulation of the "slow" motor axone of the closer muscle at approximately 20/sec. every five seconds. Injection of 0.005 B.E.'s of factor I into the claw abolishes the response of the muscle, washing (at the arrow) restores it.

FIG. 6. Kymograph records of the effects of factor I (expressed in B.E.) and acetylcholine (ach) on the heart beat of (*a*) isolated heart of the squid *Sepiotheuthis sepioidea*; (*b*) and (*c*) isolated heart of the octopus, *Octopus vulgaris*.

(4) Neuromuscular transmission in crab claws was blocked by injection of 0.005 B.E. of factor I. Only the fast and slow contractions of the closer muscle of the claw were studied. Both were affected by similar concentrations of factor I. The block was reversible (Fig. 5). These findings confirm earlier results (6). When low concentrations of factor I were used, the block could usually be overcome by stimulation with higher frequencies. Unfortunately the nature of the stimulator available did not permit quantitative evaluations and it was not possible to study the effects of factor I on nerve and muscle action potentials. Further experiments and studies on the effect of factor I on neuromuscular transmission are therefore necessary and planned.

(5) The effect of factor I on the isolated heart of squid and octopus has a striking resemblance to the action of acetylcholine in the same organs. There is no doubt that the effect of the preparation of factor I used was due to a substance other than acetylcholine since the acetylcholine content of this preparation (as determined on the sea cucumber muscle and leech muscle) was much too low to have any effect on these hearts. Whereas 0.001 B.E.

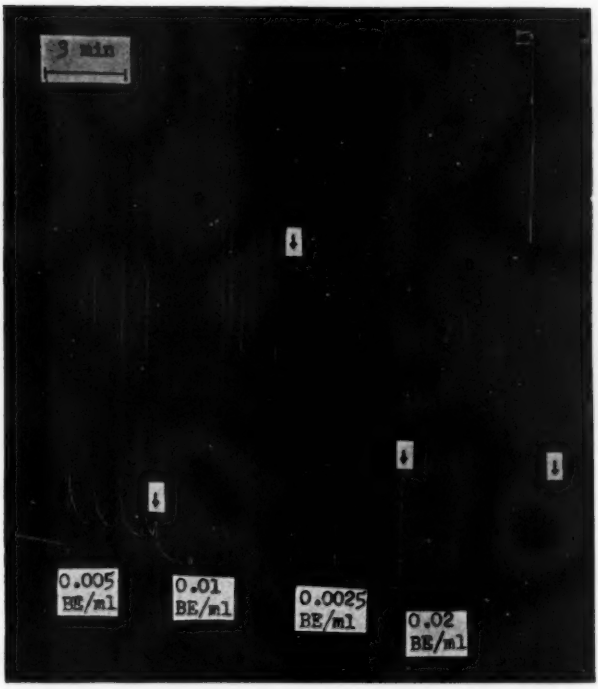

FIG. 7. Isolated rectum of *Sepiotheuthis sepioidea*. Kymograph record of the contractions induced by various concentrations of factor I (expressed in B.E. per ml.). At the arrows the preparation was washed.

of factor I stopped the hearts completely in diastole the same effect required 0.1 μgm. of acetylcholine (Fig. 6) and amounts of acetylcholine smaller than 0.05 μgm. were always found to be ineffective.

(6) On the isolated rectum of squid and octopus, factor I caused contractions which were spontaneously repeated at intervals which are determined by the concentration of factor I (Fig. 7). The lowest effective concentration of factor I was found to be 0.002 B.E./ml. Acetylcholine, even in high concentrations such as 10 μgm./ml. did not seem to have any effect except in a few cases where such high concentrations caused a slow and transient weak contracture. It was however found that rather small amounts of acetylcholine, e.g. 0.1 μgm./ml., were sufficient to block any effect of factor I completely. Fig. 8 gives an example. Atropine (10^{-7}, 10^{-6}) had no blocking effect.

It is interesting to note, that 5-hydroxytryptamine (as the creatine sulphate) had a stimulating action on the cephalopod rectum. This action of 5-hydroxytryptamine differs however from that of factor I in that it causes only one quick contraction. Concentrations lower than 10^{-6} were found to be ineffective.

(7) On the isolated dorsal muscle of the leech no specific effect of factor I could be discovered. The presence of a small amount of acetylcholine in the

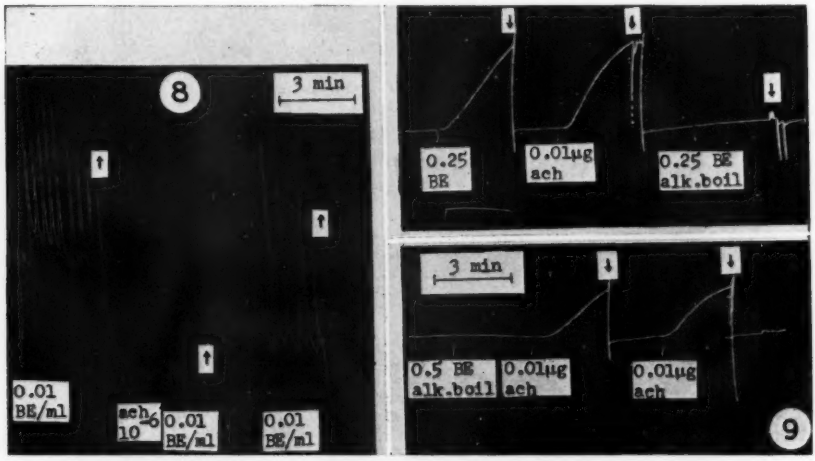

FIG. 8. Isolated rectum of *Sepiotheuthis sepioidea*. Kymograph record showing the blocking effect of acetylcholine (ach) on the action of factor I (expressed in B.E. per ml.). At the arrows the preparation was washed.

FIG. 9. Isolated back muscle of the leech, *Hirudo medicinalis*. Kymograph record of the contractures induced by acetylcholine (ach) and a preparation of factor I (expressed in B.E.). Some of the latter preparation was boiled with alkali (alk. boil) and had no effect.

preparation of factor I could however be detected. The estimated concentration was of the same order of magnitude as that which acted on the sea cucumber muscles (see above). Three minutes boiling at pH 11 (NaOH) destroyed the acetylcholine activity of our factor I preparation completely (Fig. 9). Factor I is not affected by this treatment (4).

Discussion

The present study adds seven more to the number of physiological preparations on which the effects of factor I have been studied. There seems little doubt that factor I has no effect on the longitudinal muscles of sea cucumber and leech and that, in these organs, it does not interfere with the action of acetylcholine. A similar situation has been found with mammalian striated muscle (10). On the other hand previous findings concerning neuromuscular block in the crayfish (*Cambarus clarkii* Girard) have been extended and confirmed in several species of crabs. The interaction of acetylcholine and factor I on crustacean muscle has not been studied since it is uncertain whether acetylcholine is involved in crustacean neuromuscular transmission. The same applies to the spine reflexes of sea urchins which are inhibited by factor I.

A definite antagonism between factor I and acetylcholine has been established in the crustacean heart, intestine (6, 7), and stretch receptors (6). Such antagonism is most likely also responsible for the action of factor I in mammalian sympathetic ganglia where it blocks synaptic transmission (10). This antagonism was also found in the cephalopod rectum where acetylcholine blocks the effect of factor I.

There are, however, several physiological preparations in which the action of factor I solutions resemble that of acetylcholine. The similar effect of factor I solutions and acetylcholine on the hypoglossal nucleus of the cat has already been described (11). A like situation has now been found in the cephalopod heart, where both have a strikingly similar inhibitory action. To what extent the excitatory action ascribed to factor I in polysynaptic spinal reflexes of the cat (11) is due to a synergistic or similar action of factor I and acetylcholine cannot be decided.

From all these facts one is tempted to speculate that in many physiological preparations acetylcholine and factor I might compete for the same receptor structure and that they may therefore possess a chemical similarity. The fact that acetylcholine blocks the stimulatory action of factor I on the cephalopod rectum is especially striking in this respect.

It has been suggested (6, 10, 11) that factor I might be the transmitter substance of inhibitory neurons in the mammalian central nervous system and of central and peripheral inhibitory neurons in decapod crustaceans. The following evidence was brought forward:

(1) Factor I occurs only in the central nervous system and is absent in peripheral nerves of mammals. Its localization in the nervous system therefore corresponds roughly with that of inhibitory neurons.

(2) It is able to block synaptic transmission in monosynaptic spinal reflexes of the cat.

(3) This effect is prevented by subconvulsive doses of strychnine, which corresponds very well with the findings of Bradley, Easton, and Eccles (3) that subconvulsive doses of strychnine prevent the action of inhibitory neurons in the same reflex pathways.

(4) Factor I imitates the action of inhibitory neurons in the heart, stretch receptors, and neuromuscular transmission of the crayfish (5).

(5) It is inactivated by nervous tissue (6).

(6) It is produced by the living brain (10).

(7) It counteracts the action of acetylcholine in a number of physiological preparations and might therefore be able to inhibit central synaptic transmission where acetylcholine is involved as transmitter.

(8) It is without effect on mammalian neuromuscular transmission, a site where no inhibitory neurons are involved.

The present study offers some more arguments in favor of factor I being a transmitter substance of inhibitory neurons. The ineffectiveness of factor I on the sea cucumber and leech muscles could very well be a parallel to the situation in mammalian neuromuscular transmission. This would mean that in those cases where no peripheral inhibitory neurons are involved there is no receptor structure present on which an inhibitory transmitter could act. On the other hand, the inhibitory action of factor I on reflexes of sea urchins could be compared with the effect of factor I on mammalian monosynaptic reflexes. There can be little doubt that neuronal inhibition is involved in the spine reflexes of sea urchins (15) and one can picture factor I as acting at the same

synaptic sites at which inhibition is normally taking place. A factor, so far indistinguishable from factor I in its effect, has been found in peripheral nerve of crustaceans. This is another instance where the presence of inhibitory fibers is accompanied by the presence of factor I.

On the other hand, the present study as well as previous results gives evidence which is difficult to fit into a picture in which factor I plays the role of a transmitter substance of inhibitory fibers. We must omit the mammalian sympathetic ganglia from this discussion because there is a possibility that inhibitory neurons are involved in their function (12). The excitatory actions of factor I on the hypoglossal nucleus and on polysynaptic reflexes of the cat certainly indicate that factor I is not necessarily connected only with inhibitory neurons. This is now further demonstrated in the case of the cephalopod rectum where factor I has a powerful stimulating effect. There are other physiological preparations, such as the cephalopod heart and rectum, where there is no evidence that factor I is normally involved but at which factor I solutions exert a rather powerful action. As mentioned above, however, there is suggestive evidence that factor I and acetylcholine compete for the same receptor substrate. This does not necessarily mean that either of them normally acts on this substrate, although it is likely that acetylcholine is involved in synaptic transmission in the hypoglossal nucleus (13) of the cat and in the cephalopod heart (2). It is a well known fact that various cholinergic drugs differ in their effects with respect to the physiological preparation to which they are applied. One substance may be excitatory in one preparation, inhibitory in another, and ineffective in a third. The concept of muscarinic and nicotinic actions cannot longer be maintained but it is obvious that differences in the substrate (the receptor structure) are at least as important as the differences in the structure of the cholinergic compounds. So far it seems possible to classify the active principle of factor I as cholinergic in spite—or because—of its antiacetylcholine activity in certain preparations.

In a number of preparations in which it blocks the excitatory action of acetylcholine, factor I has an inhibitory effect on its own. This is true for the crayfish stretch receptors (6), the rabbit ileum (5), and the crayfish intestine (6, 7). In the latter two organs atropine has a similar inhibitory effect but in the stretch receptors atropine induces a frequency increase although it blocks the effect of the much more potent acetylcholine. In this case it is possible that factor I counteracts acetylcholine because of its direct inhibitory action rather than by blocking acetylcholine. It seems probable that even an inhibitory transmitter substance would have to act on a receptor structure and it could very well be that in certain cases a cholinergic receptor structure is receptive for an excitatory (acetylcholine) as well as an inhibitory factor.

Until a final isolation of the active substance(s) of factor I is achieved none of the actions of this factor can be definitely ascribed to one single substance. At present, however, it seems satisfactory to assume that there is only one active substance responsible for the effects of factor I, since all the known biologically active substances of brain are practically excluded in the course of

purification of factor I. This is especially true for histamine, adrenaline, noradrenaline, hydroxytryptamine, ATP, ADP, thiamine, substance P, ammonium ions, and acetylcholine, which substances are precipitated or destroyed by perchloric acid or lead acetate.

Acknowledgments

I wish to express my thanks to Dr. L. W. Krumholz who did everything to make my stay at Bimini a most pleasant and successful one. I also want to thank Dr. K. A. C. Elliott for his continuous interest and encouragement and for critically reading the manuscript.

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PAROTID SECRETION OF IODIDE[1]

By Marion H. Ferguson, A. Naimark,[2] and J. A. Hildes

Abstract

Parotid juice from normal human subjects was collected by means of suction cups over the parotid papillae. The iodide content of the secretion was determined at various flow rates with and without the oral administration of potassium iodide, ammonium thiocyanate, and methimazole (Tapazole, Lilly), as these drugs are known to influence iodide metabolism in the thyroid gland. An inverse curvilinear relationship was found between the concentration of iodide and the rate of parotid secretion. Potassium iodide by mouth increased the concentration of parotid juice iodide in the same proportion as the increase in blood level of iodide. The amount of iodide secreted by the parotid glands was depressed by the administration of thiocyanate but was not influenced by the administration of methimazole.

Introduction

The presence of iodide in saliva has been recognized for over fifty years (6). Recent interest has been stimulated by reports suggesting similarities between the thyroid and salivary glands in the metabolism of iodide. Myant *et al.* (10) found that both the gastric glands and the salivary glands concentrated radioiodine to a degree comparable to the thyroid. Rowlands *et al.* (11) have shown that this concentrating ability of the salivary glands is depressed by thiocyanate and perchlorate, both of which also depress the thyroid accumulation of iodide. Fawcett and Kirkwood (2) have reported certain enzymes in the parotid and submaxillary glands of rats which are also present in the thyroid. In the latter situation they are considered important links in the organic binding of iodine, but there is contradictory evidence of their role in the salivary glands (3, 12, 14). Thode *et al.* (13) have found a difference in salivary iodide excretion in abnormal thyroid states compared to euthyroid subjects. The significance of these findings is not clear (4).

As the rate of secretion of saliva is known to affect the concentration of other ions in saliva (8), it seemed that this aspect of iodide secretion should be investigated. Some dependence of salivary iodide excretion on rate of salivary flow was indicated by Heath and Fullerton in 1935 (7). The experiments reported here on normal human subjects show that the rate of secretion of parotid juice does influence its iodide excretion. In addition the effect of drugs, known to influence the iodine metabolism of the thyroid gland, was also tested on parotid iodide excretion, taking the rate of secretion into account.

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Contribution from the Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Manitoba. This work was aided by a grant from the National Research Council of Canada.
[2]*Scholar of the Manitoba Institute for the Advancement of Medical Education and Research.*

Methods and Materials

Experimental

All the experiments were carried out on three normal adult subjects. The subject attended the laboratory in the morning without breakfast and, when he was comfortably seated, collecting cups were fixed over both parotid papillae (9). The secretion of each gland was delivered into graduated 15 ml. tubes for varying intervals timed with a stop watch. Secretion was reflexly stimulated by applying varying strengths of dilute acetic acid, up to 1%, to the tongue. For low rates, samples of resting secretion were collected. A random order of stimulation was employed to eliminate any influence of fatigue although previous experience had indicated that this was not a factor in the rate of secretion or in the concentration of electrolytes. The duration of each stimulus period varied, depending on the flow rate, to allow collection of sufficient secretion for analysis. It was usually three to five minutes, but to collect sufficient resting secretion sometimes took 20 to 30 min. A five-minute interval was allowed between collections.

The experiments are listed in Table I. Two other preliminary tests were conducted to establish the dose of I^{131} and potassium iodide required to achieve satisfactory blood levels throughout the experimental period. From Table I it may be seen that I^{131} was given two to three hours before collection in three experiments and 750 mgm. potassium iodide in gelatine capsules was ingested two hours before collection in five experiments. There were five control periods with collection after iodide alone; there were five collection periods after 2 gm. thiocyanate in 100 ml. water given by stomach tube; and there were two collection periods after the administration of 100 mgm. methimazole (Tapazole, Lilly). The latter was given 12 to 14 hr. before the collections were started.

TABLE I

SUMMARY OF EXPERIMENTAL PROCEDURES

Expt. No.	Subject	Iodide dosage		Collection periods, hr. after administration of iodide		
		I^{131}, μc.	Potassium iodide, mgm.	Control	Thiocyanate*	Methimazole†
1	A	60 I.V.	—	2.5–4.5	5.0–8.0	—
2	B	—	750	2.5–4.5	—	—
3	B	—	750	—	2.5–5.0	—
4	B	—	750	2.0–4.0	4.0–7.0	—
5	B	—	750	—	—	3.0–5.0
6	C	—	750	3.0–5.0	5.5–8.0	—
7	C	100 orally	—	—	—	3.0–6.0
8	C	100 orally	—	2.5–4.0	5.0–6.0	—

**2 gm. thiocyanate in 100 cc. water by stomach tube 0.5 to 1 hr. previously.*
†100 mgm. methimazole ingested 13 to 14 hr. previously.

Blood samples were taken at intervals throughout each experiment for determination of stable iodine or radioiodine as indicated. In experiments 7 and 8 radioactive counts were taken six inches above the thyroid isthmus and also above the skin of the thigh two hours, four hours, and six hours after I^{131} ingestion.

Analytical Procedures

Stable iodine was estimated by Alpert's method (1) with a thiosulphate titration. Radioactivity of blood and salivary samples was estimated in a liquid counter as described by Zingg and Perry (15).

Results

The relationship of iodide concentration in saliva to the rate of salivary flow is shown in Fig. 1. The data are from the control periods of experiments 6 and 8. It can be seen that there is a curvilinear negative relationship between salivary iodide concentration and rate of salivary flow and that this relationship holds when the iodide levels are determined by carrier-free tracer dose of I^{131} as well as after the ingestion of 750 mgm. potassium iodide. The order of difference in the concentrations between these two experiments is approximately 1000 times. Similar results were obtained in all the experiments except in experiment 4 (Table I) when only "resting" secretions were collected

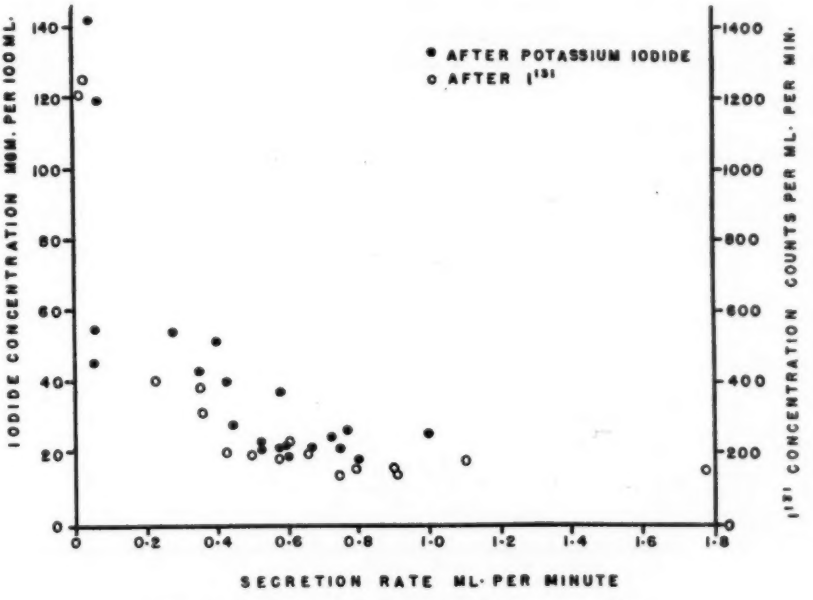

FIG. 1. Parotid secretion rate and iodide concentration.

at slow flow rates. However, the curves varied somewhat in detail—in some cases appearing to level off above secretion rates of 0.5 ml. per min., in others levelling off only above 1.0 ml. per min., and in some not levelling off at all within the range of rates studied.

The concentrating ability of the glands for iodide, as indicated by the ratio of concentration in the saliva to that in serum, is shown in Fig. 2 for the same experiments. It may be seen that the saliva: serum ratio also varies inversely with the rate of secretion, being as high as 60 at low rates of secretion in this experiment. In other experiments in this series ratios over 100 were sometimes found. The ratio falls to somewhere between 5 and 10 at fast rates of secretion. The data for both experiments (I^{131} and potassium iodide) fell along the same curve. In other words, the concentrating ability of parotid glands for iodide was not impaired by a very high blood level of iodide.

The effect of thiocyanate on parotid salivary iodide was determined in all three subjects, in one after a 750 mgm. dose of potassium iodide, in another after a tracer dose of I^{131}, and in the third subject by both methods on different occasions (experiments 1, 3, 4, 6, and 8). In all cases the results were similar and are exemplified by Fig. 3. The salivary: serum ratio of iodide is significantly depressed by thiocyanate. In the data shown the reduction in ratio is of the order of 60%. There was no systematic alteration in salivary flow rate.

The effect of methimazole, an organic sulphur compound which blocks the organic binding of iodide in the thyroid, was tested in experiments 5 and 7. The data in the latter experiment, together with control data on the same subject, are shown in Fig. 4. It is seen that the methimazole had no apparent

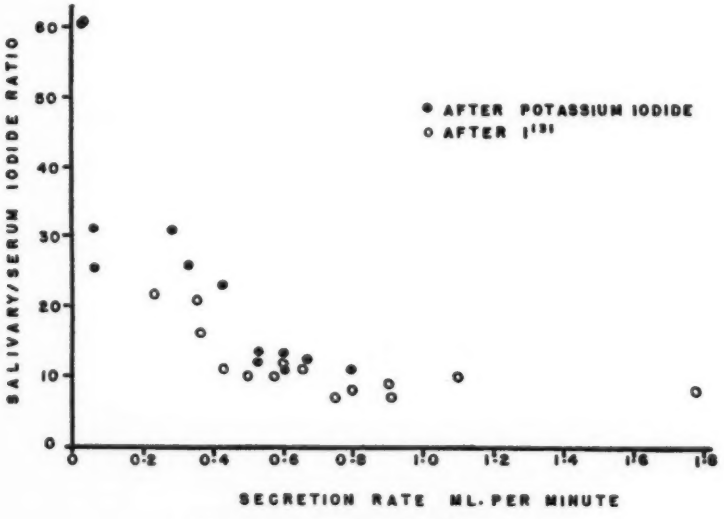

FIG. 2. Salivary/serum iodide ratio.

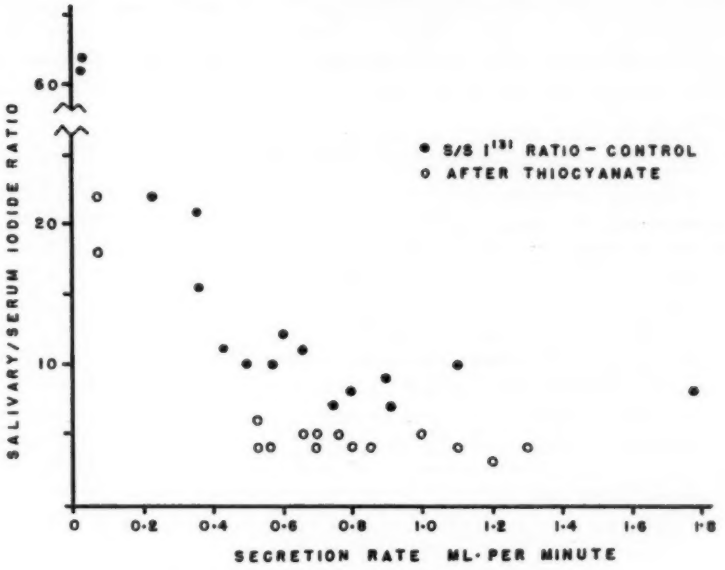

FIG. 3. Effect of thiocyanate on parotid iodide secretion.

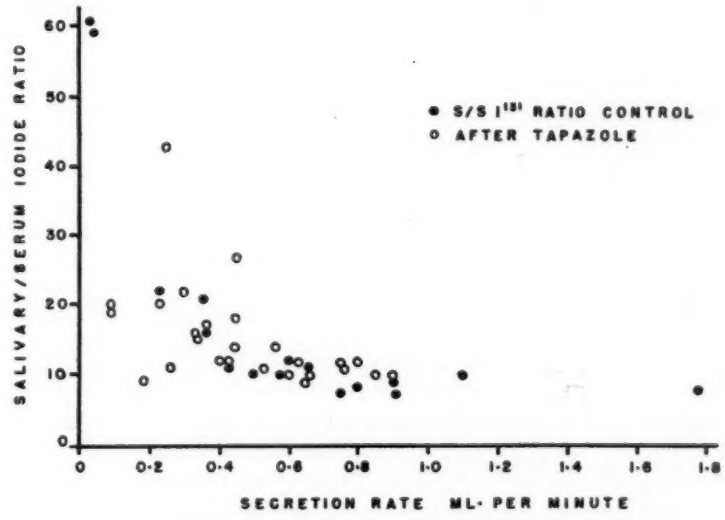

FIG. 4. Effect of tapazole on parotid iodide secretion.

influence on iodide secretion by the parotid. A similar result was obtained in experiment 5 in another subject. The 24-hr. thyroid uptake of I^{131} in experiment 7 indicated effective blocking of organic binding in the thyroid. The two-hour thyroid pickup was 6.9% of the dose but this had fallen to 2.5% by 24 hr.

Discussion

Although the results confirm that there are certain similarities between the thyroid and the parotid glands in that both concentrate iodide to a high degree and that this concentrating ability is inhibited in both by the administration of thiocyanate, certain other factors of practical importance also emerge.

The dependence of parotid iodide concentration on the rate of secretion would make it difficult to interpret changes in salivary iodide secretion in abnormal states without taking the rate of secretion into account.

It is not possible from the present results to determine whether the depression of iodide pickup by thiocyanate is of the same order in the parotid and thyroid glands. Further experiments specifically directed to this point would be interesting.

Under some circumstances the administration of large doses of iodide seems to depress thyroid gland function. The effect of Lugol's solution on hyperthyroidism has been known for many years. The mechanism of this action of iodide is still the subject of considerable experimental study (5). However, with regard to the parotid gland, the present results indicate that the iodide load over a wide range does not depress the concentrating ability of the parotid glands for iodide.

The thyroid hormone blocking agent used did not affect parotid iodide secretion although there was evidence that hormone synthesis was effectively inhibited.

Acknowledgment

We are grateful to Miss K. L. Rasmussen for technical assistance.

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THE EFFECTS OF CONVULSANT AGENTS ON PARTIALLY ISOLATED REGIONS OF THE CENTRAL NERVOUS SYSTEM[1]

By C. G. Drake, J. J. Seguin, and G. W. Stavraky

Abstract

The effects of removal of one motor cortex, a frontal lobe, or a complete cerebral hemisphere on the action of various convulsant agents were studied in chronic cats and in spinal preparations. In both sets of experiments, previously decentralized neurons responded to smaller quantities of convulsant drugs than did the intact ones, and pentylenetetrazol, camphor, and picrotoxin, as well as strychnine and acetylcholine, evoked greater and more prolonged responses from these neurons when sufficient time was allowed for sensitization due to partial isolation to take place (in the majority of experiments two to eight months). This was ascertained in myographic recordings and in studies of the electrical activity of the anterior horn cells in high spinal cats and white rats, as well as in photographic and statistical analyses of convulsions induced in chronic animals. The latter study revealed that the convulsions were asymmetrical in the operated cats, the muscular contractions being exaggerated and prolonged contralaterally to the cerebral ablation. The median convulsant dose (CD_{50}) of pentylenetetrazol for the control cats was 7.8 mgm./kgm. (95% confidence limits 7.4–8.1) while that for the operated group was 6.8 mgm./kgm. (95% confidence limits 6.3–7.2). The latent period following the injection of pentylenetetrazol was significantly shorter for the operated group than for the control one, and the convulsions lasted longer in the operated cats than in intact animals. The longer duration of convulsions in the operated group depended on a significantly longer tonic and terminal clonic phase of the convulsion. Two patterns of convulsions could be elicited—a clonic and a more severe clonic–tonic–clonic (CTC) one. In the control group clonic convulsions were more frequent at low dosages of pentylenetetrazol while CTC convulsions predominated at high dosages. In the operated group CTC convulsions were prevalent throughout the range of doses used and occurred in a greater percentage of animals than in the control group. Chemically induced convulsions play a prominent part in the study of epilepsy and in the treatment of some mental derangements, and it is felt that this investigation may contribute to the understanding of the mechanism of action of convulsant agents on the nervous system in which abnormal conditions prevail.

Introduction

Cannon and Haimovici (3) and Stavraky and co-workers (36, 37, 15, 10, 6, 40) have shown that spinal motoneurons become very sensitive to various chemical stimulating agents when they are partially isolated from their connections. On the other hand, conflicting reports continue to appear in the literature pertaining to the susceptibility of experimental animals to chemically-induced convulsions after ablations of various parts of the central nervous system. The opinion most commonly adhered to, is that such convulsant agents as absinthe, camphor, and pentylenetetrazol exert their action predominantly on the cerebral cortex and that larger quantities of these drugs are required to produce convulsions after removal of various parts of the brain. Thus, Bertha (1), Pike and Elsberg (30), Uyematsu and Cobb (44), Hahn (18), van Bork, Dalderup, and Hirschel (2), Ten Cate and Swijgman (41), and many others using different techniques and criteria came to the conclusion that, on the whole, cerebral ablations diminish the sensitivity

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of experimental animals to chemically-induced convulsions, while Sauerbruch (34) and Dandy and Elman (8, 9) maintained that the reverse was the case. In most of these investigations, the duration of the period of recovery following the cerebral ablations was not adequately considered and the treatment of the results was such that definite proofs, one way or another, were lacking. With the availability of modern statistical techniques and other methods of investigation it seemed that this problem could be reinvestigated with conclusive results and it was made the subject of the present study.

Methods

The effects of pentylenetetrazol, camphor monobromate, and some other convulsant agents were studied in a series of chronic and sacrifice experiments which were carried out on 60 cats in which various portions of the cerebral hemispheres were removed in aseptic operations: in 20 animals one frontal lobe* was removed; in 36, one cerebral hemisphere was extirpated; and in four cats, the left sensorimotor cortex (anterior and posterior sigmoid gyri) was excised. The technique and extent of the removals were similar to those described previously by Stavraky (36), the operation usually involving the left cerebral hemisphere.

Two separate experiments were carried out on the chronic animals. In the first experiment, 20 semidecerebrate, four left frontal-lobectomized, and four animals with ablations of the anterior and posterior sigmoid gyri were injected periodically with camphor or pentylenetetrazol. The injections were carried out beginning 72 hr. after the operation and continued up to seven months. The patterns of convulsions and effects of repeated injections upon them, as well as the optimal spacing of the injections, were studied and moving pictures and other photographic recordings of typical convulsions were made. In this series of experiments many animals received weekly injections of acetylcholine for varied periods of time intermittently with pentylenetetrazol and camphor. These animals seemed to develop a particular proneness to asymmetrical convulsions which became greatly exaggerated toward the end of the study. In the subsequent experiment in which statistical analysis was employed, a new group of cats was used for this reason. In this experiment, 17 semidecerebrate and 18 frontal-lobectomized animals (three carried over from the first experiment after over 12 months of rest) were injected with pentylenetetrazol. A control group of 89 normal cats was also injected with this agent and the results obtained on normal and operated groups of animals compared. The maximum of three consecutive weekly injections was set and not exceeded in the operated animals, while the maximum of four consecutive injections was given to the controls. The fourth injection given to these animals was not included in the calculation of the median convulsant dose of pentylenetetrazol. A prolonged period of rest was given before any further experiments were carried out. All the injections, the results of which were used for the

**The terms 'frontal lobectomy' and 'removal of the frontal lobe' are used interchangeably throughout and refer to a complete removal of a frontal lobe, the motor cortex being included in the ablation.*

statistical analysis, were made into the femoral vein through a 24 gauge needle and the duration of the injections was kept constant. The various phases of the convulsion were timed to the nearest second by means of stop watches; the latent period was considered to be between the end of the injection and the beginning of the twitches. Pentylenetetrazol (metrazol, Bilhuber-Knoll) 25 mgm./ml. was dissolved in distilled water; camphor monobromate (B.D.H.) was dissolved in 95% ethanol 100 mgm./ml. in accordance with the technique of Wortis, Coombs, and Pike (46). The mean durations of the studied components of the convulsions and their standard errors were calculated and differences between groups tested for significance by means of Fisher's "t" test (14); differences have been considered significant when $P < 0.05$. Also, the median convulsant dose for pentylenetetrazol was calculated from this experiment by the method outlined by Finney (13).

In addition to the chronic experiments, 37 sacrifice experiments were carried out in which myographic recordings from both quadriceps muscles were made with a technique described elsewhere (Cannon and Haimovici (3), Stavraky (36), and Drake and Stavraky (10)) and the electrical activity of the anterior horn cells was compared on the two sides of the spinal cord in recordings made from the central ends of both femoral nerves following section of the latter and curarization of the animals. The recordings were made by means of bipolar silver – silver chloride electrodes which were connected with differential balanced push–pull amplifiers. The amplified potentials were visualized and photographed on a dual beam oscilloscope. Nine experiments were performed on previously semidecerebrate or frontal-lobectomized cats and 28 following an aseptic semisection of the spinal cord, the time interval between the operations and the experiments ranging from four days to 15 months. A series of control experiments were also carried out on white rats in which the activity of the anterior horn cells was monitored from the severed sciatic nerves under experimental conditions similar to the ones described for the cats. This part of the work was done in collaboration with Mr. G. A. Bobb and in some of these experiments the recordings were done without the use of curare. Acetylcholine bromide (Eastman-Kodak), strychnine sulphate (Parke-Davis), and picrotoxin (Eimer-Amend) as well as pentylenetetrazol and camphor were tested in these experiments. The injections were done intra-aortally through a plastic catheter introduced by way of the left subclavian or external carotid artery.

Results

I. The Effects of Pentylenetetrazol on Intact and Chronic Frontal-lobectomized or Semidecerebrated Cats

A. Description of Convulsions

Intact Cats

A dose of 4 mgm./kgm. of pentylenetetrazol occasionally evoked blinking and ear twitches but no convulsions. In most of the animals, 6, 8, 10, and 12 mgm./kgm. of this agent caused any one of three types of responses: (1) A

mild convulsion which manifested itself in an excitation followed by twitching of the ears, blinking, and several generalized twitches of the extremities. Dilatationof the pupils, piloerection, and vocalization also occurred but the cat did not fall. This type of response was classed as a clonic convulsion (CL) in the statistical analysis when the clonic twitches lasted longer than six seconds, the same criterion holding in the operated group of animals. (2) A pronounced clonic convulsion (CL) started with flexion of the limbs, the animal falling to the ground and having a series of rapid extensor twitches. This was sometimes followed by a transient extensor tone and always by a period of flaccidity. During recovery, the animal lay panting and sometimes showed progression movements. (3) A clonic–tonic–clonic (CTC) type of convulsion started similarly to the previous one; but during this convulsion the initial clonic phase was followed by a period of generalized tonic flexion, during which the head was ventroflexed, the front limbs raised under the neck or over the head, and the hind limbs held rigidly semiflexed. Toward the end of the tonic period the head was maximally flexed, the hind limbs gradually extended and began to twitch, this marking the onset of the terminal clonus. Occasionally, between these two stages a transient generalized extension occurred. The clonic twitches increased in violence and spread to the rest of the body, including the forelimbs, and ended abruptly, the animal developing a transient opisthotonos and extension of all four limbs. The convulsion was followed by a period of flaccidity which was sometimes interrupted by running movements and secondary twitches. Autonomic manifestations, such as transient dilatation of the pupils followed by constriction, micturition, defecation and piloerection, as well as vocalization, accompanied the convulsions.

Operated Cats

The operated cats responded to pentylenetetrazol by a modified clonic or CTC type of convulsion. These convulsions were asymmetrical in character and their pattern depended greatly on the duration of recovery from the operation, previous injections, quantity of convulsant agent employed, and other variables. Forty-eight to seventy-two hours after the removal of a left frontal lobe or of a left cerebral hemisphere the convulsion had some clonic and tonic components on the left side but consisted predominantly of an extensor tone on the right side of the body. With prolonged recovery after the operation another type of asymmetry developed. It came on gradually and was well defined by the end of six to eight weeks, but became most pronounced four to six months after the operation. From then on, it persisted indefinitely and was observed in some animals as long as five years after the operation. Four, six, eight, and 10 mgm./kgm. of pentylenetetrazol now caused one of four types of responses: (1) A minimal convulsion, during which a rapid circling away from the side of the operation sometimes took place, or unilateral clonic extensor twitches occurred in the right extremities. When bilateral clonus developed it was more violent on the right side of the

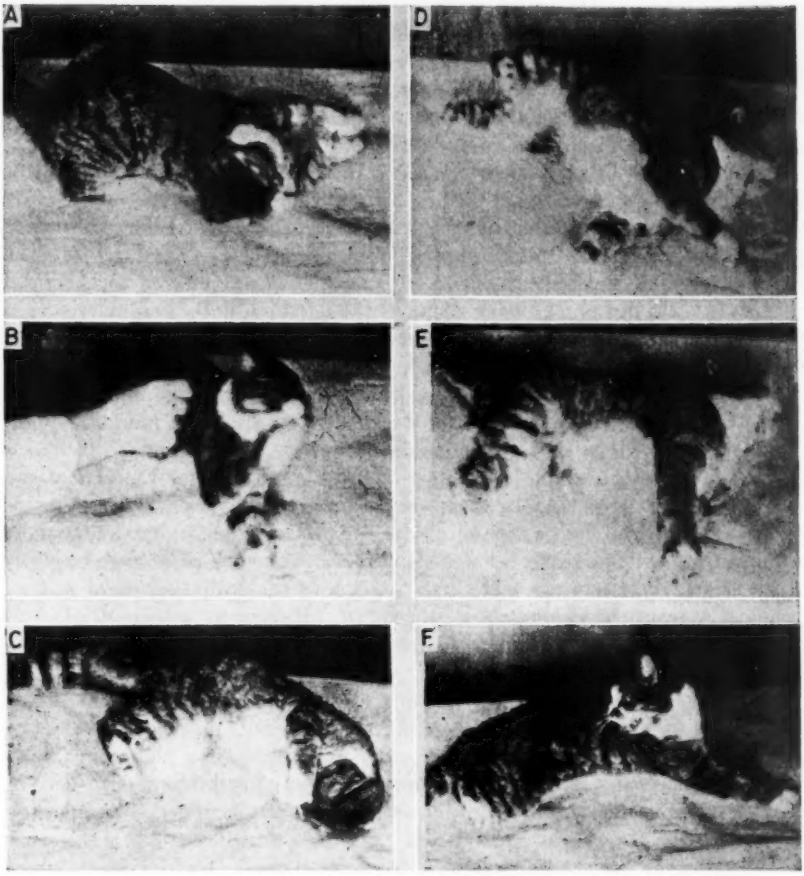

FIG. 1. Effects of intravenous injection of 8 mgm./kgm. of pentylenetetrazol in a cat one year after the removal of the left frontal lobe. A. Rolling to the right (note tonic flexion of right forelimb and protruding claws). B. End of rolling and beginning of the tonic phase. C. Tonic phase (in flexion). D. Clonic phase (note predominance of activity in the right limbs). E. Terminal extension. F. Postconvulsive hypertonicity of the right side.

body. During this type of response the cats occasionally fell or staggered to the left as if the extensor thrusts of the right extremities threw them over to this side. (2) When the convulsions were more severe the animal fell to the right with the head turned to the left and the body curved to the right, the left limbs were in clonus but the right front limb was tonically flexed. During this initial stage of the convulsion the cat rolled to the right (Fig. 1, A and B). Following this, the clonus gradually involved the right limbs and soon became more violent on the right side than on the left (Fig. 1D). During this bilateral

clonus, the rolling ceased or occasionally its direction was reversed. The clonus ended in a sudden extension of all four limbs and was followed by a short period of flaccidity and areflexia. During the recovery period, there was, at first, a hypertonicity of the right limbs, and forced grasping movements of the right forepaw were often seen. When the animal had recovered sufficiently to stand and walk, it invariably circled and leaned to the side of the lesion. (3) With a further increase in the severity of the convulsions, the initial stage was the same as in the preceding type—the animal rolled violently to the right making, on occasion, as many as 10 full turns. When the rolling ceased, the right front limb showed a transient clonus, and a stage of generalized tonic flexion ensued during which the body curved to the right (Fig. 1C). The terminal clonus began in the left hind limb, gradually spread to the right hind limb, and then to the other extremities. When fully developed the clonus was more violent on the right side than on the left (Fig. 1D). The convulsion ended in a transient opisthotonos and extension of all four limbs (Fig. 1E). After a short period of flaccidity and areflexia, marked hypertonicity of the limbs of the right side ensued and occasionally reached a stage of contracture which lasted two to three minutes after the end of the convulsion (Fig. 1F). (4) In some very severe convulsions a bilateral flexor tonus developed in the beginning of the convulsion, the flexion being most prominent on the right side. The body was curved to the right and the claws of the flexed right forepaw were often sunk into the scalp but there was no rolling. A transient clonus often intervened, followed by a second bilateral tonus, after which the convulsion followed the same pattern as the preceding one.

In a few of the animals after the end of the recovery period, secondary convulsions were seen. These occurred occasionally only on the right side, the animal walking normally on the left extremities while the right ones were clonically convulsing. Finally, the impression was gained that the severity of the autonomic manifestations e.g. dilatation of the pupils, defecation, micturition, and piloerection was greater and that these manifestations were more often seen in the operated cats than in the normal ones. A wider dilatation of the right pupil and erection of the hair only on the right side of the body were also observed. For purposes of statistical analysis the convulsions were graded as clonic or CTC judging by the pattern of responses on the fully innervated left side.

B. Effects of Increasing Doses of Pentylenetetrazol on Convulsions

As shown in Table I, when the clonic and CTC conclusions were studied separately, it became evident that increases in the quantity of injected pentylenetetrazol resulted in an increased frequency of CTC convulsions and a corresponding decrease in the percentage of clonic convulsions, a 12 mgm./kgm. dose of pentylenetetrazol giving a CTC type of convulsion in 100% of the normal animals.

Not only were the CTC convulsions more frequent both in the normal and operated animals when increasing doses of pentylenetetrazol were adminis-

TABLE I

PROPORTION OF CTC AND CLONIC RESPONSES IN RELATION TO DOSE OF PENTYLENETETRAZOL (1 INJECTION/DOSE/ANIMAL)

		Pentylenetetrazol, mgm./kgm.							
		6		8		10		12	
	Type of response	Responses/ convulsions	% with responses	Responses/ convulsions	% with responses	Responses/ convulsions	% with responses	Responses/ convulsions	% with responses
Control cats	CTC	4/9	44.4	20/44	45.5	39/61	63.9	10/10	100.0
	Clonic	5/9	55.6	24/44	54.5	22/61	36.1	0	0
Operated cats	CTC	7/10	70.0	19/27	70.4	25/32	78.1	—	—
	Clonic	3/10	30.0	8/27	29.6	7/32	21.9	—	—

tered, but the operated cats had a greater percentage of CTC convulsions than the normal animals at each dose level, and when the results for 6, 8, and 10 mgm./kgm. doses of pentylenetetrazol were pooled and compared for the control and operated cats by means of the chi-square test, a P value of <0.05 was obtained.

C. CTC Convulsions

Latent Period

Both the operated and control animals showed a gradual decrease in latency as the dose of pentylenetetrazol was increased from 6 to 10 mgm./kgm. In the controls, the reduction of the latent period was significant ($P < 0.02$) between 8 and 10 mgm./kgm. doses of pentylenetetrazol. In the operated group of animals, a significant decrease ($P < 0.01$) in duration of the latent period took place between 6 to 8 mgm./kgm. doses and was equal to almost one second. Another one second decrease in latency occurred between 8 and 10 mgm./kgm. doses ($P < 0.001$). A comparison of the latent period in the two groups of animals at each dose level disclosed no difference at the 6 mgm./kgm. level, but the operated cats had significantly shorter latencies ($P < 0.01$) at the 8 and 10 mgm./kgm. dose levels than did the controls. When the latencies for all three dose levels were combined in the control and in the operated cats, and the two groups again compared, the operated animals had a latency of 5.6 ± 0.18 sec. as against 6.6 ± 0.22 sec. in the intact animals, the P value for the difference between the two groups being <0.001. In order to make the comparison valid in this instance the number of cats in the control and operated groups was equalized at each dose level using a random selection. This procedure was adhered to in all subsequent comparisons in which the results for different dose levels were combined. Pooling of the results obtained with different doses was done only when the trends were in the same direction at each dose level. These and all the subsequent data pertaining to time relations of the pentylenetetrazol-induced convulsions are represented in Table II and Fig. 2.

Initial Clonic Period

In both the intact and operated groups of cats there was a wide variation n duration of the initial clonic phase. The intact animals showed some tendency towards a reduction in duration of this phase with increasing doses of pentylenetetrazol, but because of the wide variations this was not statistically significant. The operated group showed no persistent trend. When the results of 6, 8, and 10 mgm./kgm. doses were combined, the duration for the initial clonic period was found to be almost the same ($P > 0.4$) in the two groups, that of the intact cats being 7.1 ± 0.44 sec. and that of the operated ones 6.7 ± 0.35 sec.

Tonic Period

There was no significant change in the duration of the tonic period in either group of cats as the dose of pentylenetetrazol was increased. However, the operated group of cats had a consistently longer period than did the intact

TABLE II

MEAN DURATION (SEC. ± S.E.M.) OF VARIOUS PHASES OF THE CONVULSION IN CONTROL (C) AND OPERATED (O) CATS WITH VARIOUS DOSES OF PENTYLENETETRAZOL

Pentylenetetrazol, mgm./kgm.	Type of convulsion	Control or operated group	No. of convulsions	Latent period	Initial clonus	Tonus	Terminal clonus	Duration of convulsion
6	CTC	C	4	7.3 ± 0.95	7.8 ± 2.18	11.0 ± 0.71	11.8 ± 1.88	30.5 ± 2.66
		O	7	6.9 ± 0.35	6.9 ± 1.03	14.1 ± 2.52	22.4 ± 3.96	43.4 ± 4.10
	Clonic	C	5	6.4 ± 0.25				43.2 ± 5.10
		O	3	7.7 ± 0.33				55.3 ± 1.85
8	CTC	C	18	7.1 ± 0.35	7.3 ± 0.52	11.3 ± 0.51	15.3 ± 2.18	33.9 ± 2.10
		O	19	6.0 ± 0.10	7.9 ± 0.68	15.5 ± 1.28	19.6 ± 2.11	43.0 ± 2.14
	Clonic	C	23	6.8 ± 0.30				38.7 ± 3.99
		O	8	6.6 ± 0.33				54.4 ± 5.20
10	CTC	C	39	6.1 ± 0.20	6.4 ± 0.40	12.2 ± 0.85	14.8 ± 1.54	33.4 ± 1.38
		O	23	5.0 ± 0.25	5.9 ± 0.33	13.6 ± 0.66	19.2 ± 1.82	38.7 ± 1.53
	Clonic	C	22	6.2 ± 0.27				36.3 ± 3.38
		O	5	6.2 ± 0.37				43.0 ± 7.77
12*	CTC	C	10	5.6 ± 0.16	5.9 ± 1.13	14.2 ± 1.28	14.0 ± 2.30	34.1 ± 2.78

**Operated cats not injected with 12 mgm./kgm. dose in order to avoid any possible damage.*

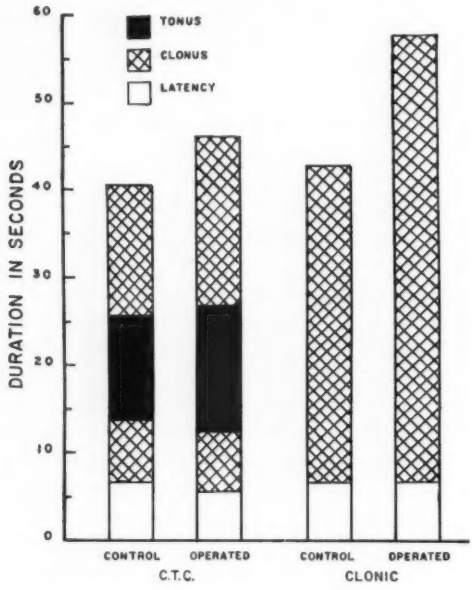

FIG. 2. Responses to pentylenetetrazol. Duration of the various phases of clonic–tonic–clonic (CTC) and clonic convulsions compared in control and operated cats (combined results of 6, 8, and 10 mgm./kgm. doses).

animals at each dose level. The variation in the duration of this phase of the convulsion was considerable and, for this reason, the difference was significant ($P < 0.01$) only at the 8 mgm./kgm. level. When the results of 6, 8, and 10 mgm./kgm. doses were combined, the operated cats had a mean tonic period of 14.5 ± 0.67 sec. while in the intact animals it equaled 12.0 ± 0.35 sec., the P value for the difference between the two groups being <0.01.

Terminal Clonic Period

The operated cats had a consistently longer terminal clonus than the intact animals at each dose level. This was shown to be significant ($P < 0.02$) if the results of the three dose levels were combined. Namely, the mean duration of the terminal clonus in the operated cats was 19.4 ± 1.47 sec. and in the intact animals 14.8 ± 1.41 sec. Owing to the variability of this phase of the convulsion, no significant trend could be detected in either the operated or the intact group of animals as regards its duration at different dose levels of pentylenetetrazol.

Total Duration of the CTC Convulsions

The total duration of CTC convulsions showed little change with increasing doses of pentylenetetrazol over the range of doses employed, but a comparison

of the two groups of animals showed that the duration of convulsions was significantly longer in the operated group than in the normal group at the 8 mgm./kgm. dose ($P < 0.01$) and at the 10 mgm./kgm. dose ($P < 0.02$). When the results of the three dose levels were combined the mean duration for the operated cats was 40.7 ± 1.28 sec. and for the intact animals 33.9 ± 1.33 sec., the P value for the difference between the two groups being < 0.001.

D. Clonic Convulsions

Latent Period

The control group of cats showed no significant change in duration of this period with increasing doses of pentylenetetrazol. On the other hand, in the operated animals a significant reduction ($P < 0.05$) in the duration of the latent period did take place between the 6 and 10 mgm./kgm doses. In comparing the latencies in the control and operated cats, it was found that the operated animals had a significantly longer latency ($P < 0.05$) than did the intact ones at the 6 mgm./kgm. dose level of pentylenetetrazol, while at the 8 and 10 mgm./kgm. dose levels, the latencies were identical.

Duration of Convulsions

In both the control and operated cats the clonic convulsions became shorter as the dose of pentylenetetrazol was increased. On the other hand, in the operated group of cats, the clonic convulsions lasted longer than in the intact cats. This was true at each dose level and when the results of 6, 8, and 10 mgm./kgm. doses were combined the operated cats had a mean convulsion time of 51.0 ± 2.89 sec. as compared to that of 36.2 ± 4.69 sec. in the intact cats, the difference between the two groups being significant ($P < 0.02$).

The duration of the clonic convulsion seemed, on the whole, to be longer than that of the CTC type of convulsion, this being true in both control and in operated animals. In the normal group of cats the difference was significant ($P < 0.05$) at the 8 mgm./kgm. dose level.

E. Comparison of the Convulsability of the Operated and Control Cats

From the results accumulated during the study of the types and durations of various phases of convulsions, the median convulsant dose of pentylenetetrazol was calculated. Several conditions in the calculation of the CD_{50} were observed. The results of the first injection per dose per animal were used. Successive injections were spaced at least one week apart and not more than three injections in a series were employed. One of the main requirements of this method is that there shall be no cumulative effect of a drug over several doses. This was tested and it was found that with three repeated weekly injections of 6 mgm./kgm. of pentylenetetrazol, 16 operated cats showed no cumulative effects, a similar condition prevailing in the normal animals. The results of the first and third injections for the operated animals are given in the following table.

TABLE III

First injection	Third injection: Convulsed	Third injection: Not convulsed	Total
Convulsed	4	1	5
Not convulsed	2	9	11
Total	6	10	16

These figures were tested by the exact method for the chi-square (Finney (12)) since the numbers in each cell are too small for the usual method. A *P* value of 0.036 was obtained, representing the likelihood of the "convulsers" and "non-convulsers" on the first injection differing by chance alone as much on the third injection as they were observed to do. In other words, non-convulsers were not converted into convulsers by three weekly injections of pentylenetetrazol. Usually the injections were given in an ascending order (6, 8, and 10 mgm./kgm. of pentylenetetrazol); in some normal animals, however, the initial injection was 8 mgm./kgm. of pentylenetetrazol. This was followed by either a 6 or a 10 mgm./kgm. dose (this accounts for a relatively large number of normal cats which received 8 mgm./kgm. of pentylenetetrazol as compared with the groups which were injected with 6 and 10 mgm./kgm.—Table IV). In the frontal-lobectomized and semi-decerebrated groups, the majority of the cats were operated upon two to eight months before the experiment, but three animals included in this group had been operated on over 40 months previously. Thus neither the normal nor

TABLE IV

CONVULSABILITY OF CONTROL AND OPERATED CATS WITH INCREASING DOSES OF PENTYLENETETRAZOL (1 INJECTION/DOSE/ANIMAL. INCLUDES CTC AND CLONIC RESPONSES)

		Pentylenetetrazol, mgm./kgm.					
		6		8		10	
		Injections	Convulsions	Injections	Convulsions	Injections	Convulsions
Control cats	No.	64	9	87	44	70	61
	%		14.1		50.6		87.1
Operated cats	No.	34	10	34	27	33	32
	%		29.4		79.4		97.0

TABLE V

COMPARISON OF THE CD_{50} OF PENTYLENETETRAZOL (MGM./KGM.) IN CONTROL AND OPERATED CATS*

	Control cats	Operated cats
CD_{50}	7.8 (7.4– 8.1)	6.8 (6.3– 7.2)
Regression coefficient	10.0 (7.6–12.4)	11.2 (7.3–15.1)

**Values in parentheses ( ) represent 95% confidence limits.*

the operated group of animals were theoretically completely homogeneous, but this did not seem to affect unduly the results.

As in the preceding observations the results obtained on the frontal-lobectomized and on the semidecerebrate animals were pooled when the CD_{50} was calculated for the control and for the operated animals. In the calculation of the CD_{50} the chi-square test for heterogeneity of the frontal-lobectomized and semidecerebrated cats equalled 0.3 at one degree of freedom indicating that the convulsability of the two groups of animals was not significantly different ($P > 0.5$). This and direct observations of the patterns of convulsions justified the treatment of the two groups as a single unit throughout the analysis.

The relative convulsability of the operated and control animals is shown in Table IV. The comparison of the CD_{50} for the two groups of cats is presented in Table V. It indicates that the median convulsant dose had a maximum likelihood estimate of 7.8 mgm./kgm. of pentylenetetrazol for the control cats and one of 6.8 mgm./kgm. for the operated animals, the values for the two groups being significantly different.

II. Effects of Camphor

Camphor monobromate has been extensively used as a convulsant agent. Thus it was interesting to compare its effects with those of pentylenetetrazol. The alcoholic solution of camphor, however, was injurious to the veins and the site of the injection had to be changed on repeated administration of this drug. Also secondary convulsions were frequently seen after injections of camphor; due to this no attempt was made to analyze the data statistically. On the whole, the observations of Wortis, Coombs, and Pike (46) as to the quantity of camphor required to produce convulsions (3.3 to 6.1 mgm./kgm. for normal cats) were confirmed. The impression was also formed that operated cats were more sensitive to camphor than were intact animals; in two semidecerebrate animals convulsions were produced repeatedly with as little as 2.4 mgm./kgm. of camphor. In type, the convulsions resembled the ones induced by pentylenetetrazol, but camphor seemed to bring out more prominently the tonic components and in the operated animals the asymmetry of the convulsion became very marked (Fig. 3).

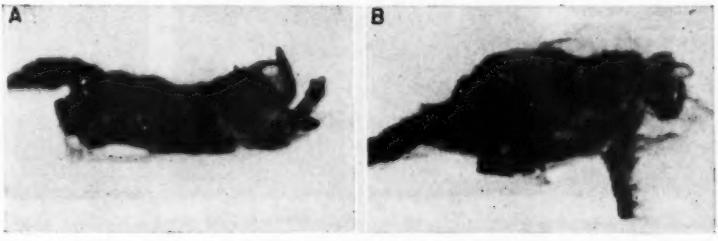

Fig. 3. Effects of an intravenous injection of 3.5 mgm./kgm. of monobromated camphor in a cat nine months after the removal of the left frontal lobe. A. Beginning of convulsion. B. Tonic phase.

III. Analysis of the Effect of Convulsant Agents

1. Experiments in Spinal Cats with Preceding Semisection of the Spinal Cord at T_{12}–L_1 Level

A. Acetylcholine, Strychnine, and Picrotoxin

Recording myographically, the results of Cannon and Haimovici (3) were completely confirmed in that both acetylcholine and strychnine stimulated more readily the spinal centers on the semisected side.

Acetylcholine was injected 26 times in various experiments in doses ranging from 0.1 to 1.0 mgm./kgm. As shown in Fig. 4, the contractions of the quadriceps were brought about by smaller quantities of acetylcholine and were greater on the side of the semisection. This effect was observed 4 to 67 days after semisection of the spinal cord, the difference in the sensitivity of the two sides becoming more marked when the interval between the semisection and the acute experiment was longer. The characteristic response consisted of a single or a double contraction, occasionally with a few twitches of the muscle supervening. These contractions came on after a shorter latent period (two to eight seconds) than in the case of strychnine or picrotoxin, often started first on the semisected side, and outlasted the ones on the control side. In view of the finding of Cannon and Haimovici (3) that acetylcholine caused a greater contraction of the quadriceps on the side of the preceding semisection of the spinal cord after section of the femoral nerves ("penultimate sensitization"), in control experiments the nerves were severed and the electrical activity of the anterior horn cells recorded bilaterally from the central ends of the two nerves. In Fig. 6 is presented a recording from such

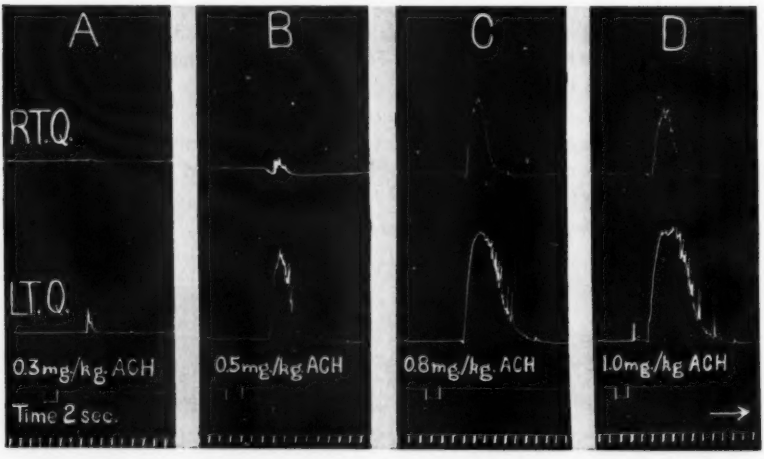

FIG. 4. Effects of increasing quantities of acetylcholine in a cat nine days after left spinal semisection at T-12 level. (RT.Q., right quadriceps; LT.Q., left quadriceps. Isotonic myograms.) In this and all subsequent experiments injections are carried out intra-aortally in high spinal preparations. Note lower threshold and greater effects on the side of the semisection.

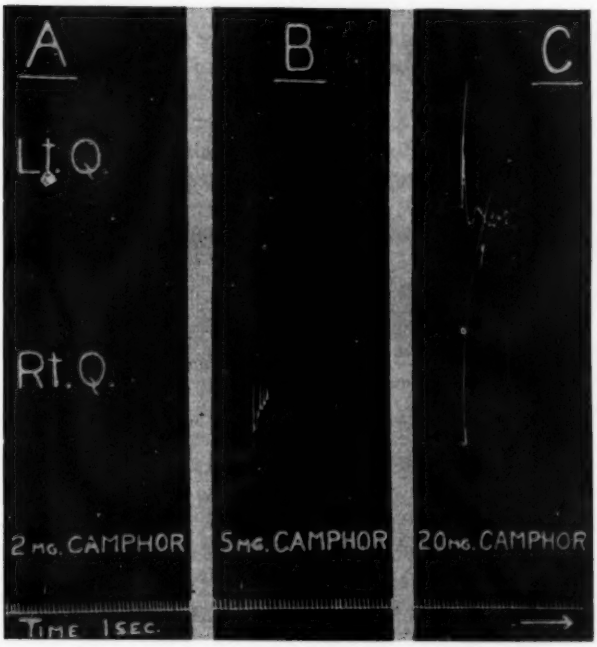

FIG. 5. Effects of camphor monobromate in cat (2.3 kgm.) 10 days after right semisection at T-12.

an experiment on a white rat 25 days after spinal semisection. Before the injection of acetylcholine the electrical activity in the sciatic nerves was equal on both sides. Three seconds after the beginning of the injection the activity increased on the semisected side and in nine seconds reached its maximum. The increase in activity on the control side came on later, was much less pronounced, and lasted a shorter period of time than on the semisected side. In Fig. 11 a similar effect of 0.3 mgm./kgm. of acetylcholine can be seen in a curarized cat in which it caused a selective discharge of the anterior horn cells on the isolated side. These experiments proved conclusively that acetylcholine stimulated the previously denervated spinal neurons to a greater degree than the ones on the opposite side of the spinal cord.

Strychnine was injected 20 times in 16 experiments in doses ranging from 0.025 to 0.5 mgm. per injection. Though introduced intra-arterially, the latent period following the injection of strychnine was relatively long ranging from 20 sec. to two minutes. Small doses (0.01–0.02 mgm./kgm.) stimulated selectively the partially isolated neurons as was shown by a series of contractions of the quadriceps muscle on the previously semisected side. Larger quantities of this agent (up to 0.1 mgm./kgm.) caused a prolonged series of contractions bilaterally which began on the operated side, were, at first, of greater magnitude and frequency, and persisted longer than on the opposite

side. Very large quantities of strychnine were seen, on occasion, to cause a reversal of this effect. Namely, after a while the contractions on the denervated side became of a lesser amplitude than those on the control side. In spite of its predominant action on the bulbar structures, *picrotoxin*, which was injected seven times in four different experiments, gave essentially similar results to those of strychnine. In quantities ranging from 0.2 to 0.6 mgm./kgm. it caused, after 20 to 40 sec. latency, more pronounced and longer lasting effects on the side of the semisection, the threshold for it being also lower on that side.

B. Camphor and Pentylenetetrazol

Camphor and pentylenetetrazol are classed as cerebral irritants. In the quantities, and with the technique used, both these agents exhibited a marked stimulating effect on spinal motoneurons. This made possible an analysis of their action on partially isolated spinal centers. Following semisection of the spinal cord, camphor was injected 12 times in 10 different experiments. Its effect was characterized by a short latent period (one to five seconds) and by a rapid contraction of the quadriceps muscles. As shown in Fig. 5, an intra-arterial injection of 0.6 mgm./kgm. of camphor caused, after a latent period of five seconds, a small contraction of the quadriceps only on the side of the semisection. An injection of 2 mgm./kgm. of camphor caused a greater contraction of this muscle after a three second latency with a suggestion of a contraction on the opposite side, while an injection of 7 mgm./kgm. of camphor resulted in a bilateral response following a one second latency, the response being greater and lasting longer on the semisected side.

Pentylenetetrazol was injected 12 times in eight different experiments. Its effect was not unlike that of camphor but was more prolonged, lasting up to two minutes on the denervated side. Following injections of pentylenetetrazol, clonic contractions developed more frequently than after injections of camphor. The latent period was also longer than after injections of camphor, usually extending from 4 to 20 sec. It was reduced when larger doses of the drug were injected and was shorter on the side of the semisection. Small doses of pentylenetetrazol (8–10 mgm./kgm.) caused selective contractions of the quadriceps on the side of the semisection with little or no effect on the control side. Larger quantities of pentylenetetrazol (10–30 mgm./kgm.) caused marked bilateral series of contractions of the muscles which started earlier on the sensitized side and lasted longer on that side (Fig. 8). Very large quantities of pentylenetetrazol (30 mgm./kgm.) were seen to cause an initial relaxation of the muscles which lasted four or five seconds, was more pronounced on the semisected side, and was succeeded by a series of bilateral contractions. Electrical recordings from severed femoral or sciatic nerves showed that pentylenetetrazol, similarly to acetylcholine, caused a greater increase of activity on the semisected side; the increased electrical activity became apparent first on that side and continued longer than on the control side. It was also brought about by smaller quantities of pentylenetetrazol.

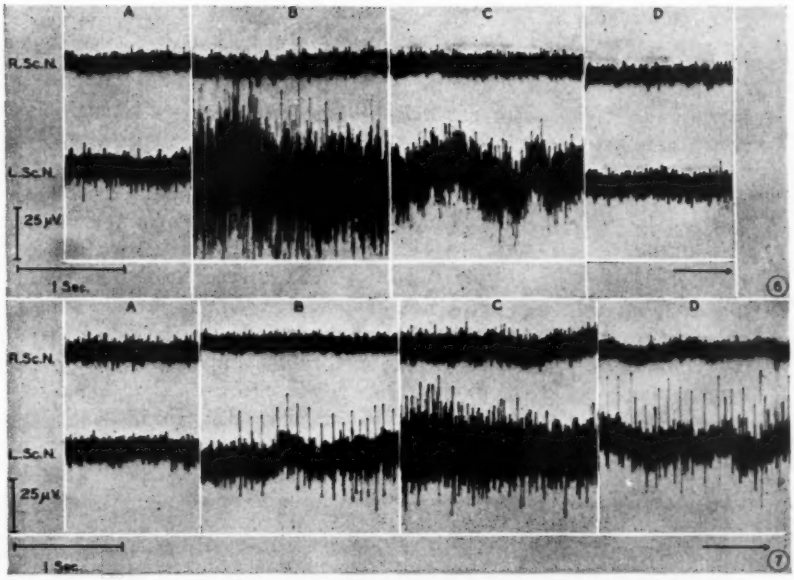

FIG. 6. Effects of 0.09 mgm./100 gm. of acetylcholine in a white rat 25 days after left semisection of spinal cord at T-12 level. Oscilloscopic recordings from the central ends of severed sciatic nerves. (R.Sc.N., right sciatic nerve; L.Sc.N., left sciatic nerve.) A. Shows activity three seconds after the start of the injection (injection time, two seconds)—beginning of increase in activity of the left side but normal activity on the right. B. Activity nine seconds after start of the injection—maximal effect of acetylcholine. C. Activity 16 sec. after start of the injection—subsiding, but still increased on both sides. D. Activity 39 sec. after start of the injection—that on the right side returned to the preinjection level, while that on the left side is still somewhat increased.

FIG. 7. Effects of 0.23 mgm./100 gm. of pentylenetetrazol in same preparation as in Fig. 6. A. Control taken one hour before the injection. B. Activity 21 sec. after the start of the injection (injection time, 7.5 sec.). Note increase in activity on left side but not on the right. C. Activity 55 sec. after the start of the injection. Maximal effect—more marked on the left side. D. Activity 75 sec. after the start of the injection. Effect persists on the left side but normal activity on the right side.

An analysis of these effects was carried out on five Wistar rats 10–36 days after semisection of the spinal cord (Fig. 7). After the injection of 0.2 to 2.0 mgm. of pentylenetetrazol per 100 gm. of rat the mean increase in electrical activity of the lower motoneurons on the intact side was found to be 13 μv., while on the side of the semisection it was 22 μv. The difference between the values was statistically significant ($P < 0.05$).

C. The Circulatory Effects of the Drugs Employed and Their Relation to the Convulsant Action

The relationship between the vasomotor and convulsant effect of epileptogenic drugs was studied by Hall (19), Hahn (18), and others. Particularly, the effect on the systemic blood pressure was often associated with the convulsant action of various drugs. For this reason, blood pressure records were made in control experiments in which convulsant agents were injected. Owing to the fact that all the injections in sacrifice experiments were carried out intra-arterially, the variations of the blood pressure level were not great except in the case of camphor. Acetylcholine often caused a slight rise of blood pressure coincidental with the contraction of the quadriceps, this being followed by an insignificant lowering of blood pressure. Strychnine had almost no effect in this respect, slight variations in the blood pressure level being readily accounted for by the muscular contractions. On the other hand, camphor had a pronounced effect causing a prompt rise of blood pressure

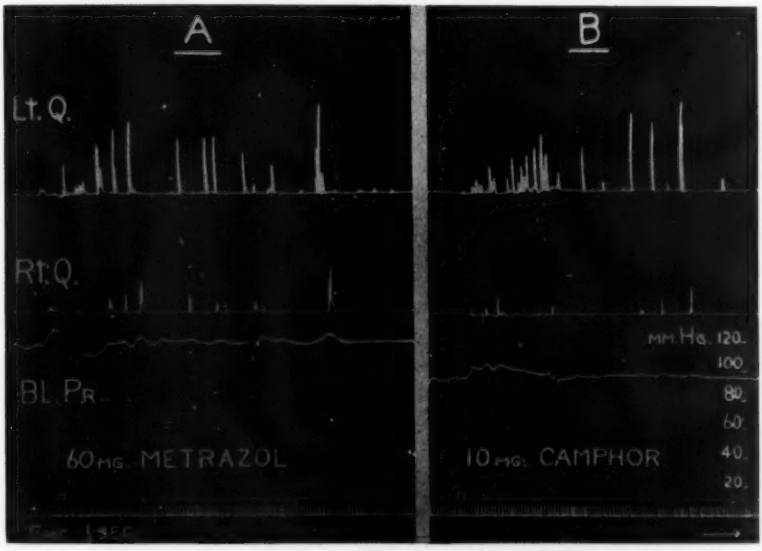

FIG. 8. Effects of pentylenetetrazol and camphor in cat (2.4 kgm.) eight days after left semisection of the spinal cord at T-12. Blood pressure controlled by means of a mercury valve.

which persisted up to two minutes following the injections, while pentylenetetrazol had a less pronounced pressor effect and when injected after camphor often caused a lowering of blood pressure instead of a rise. However, both in the case of camphor and of pentylenetetrazol, as well as with the other drugs employed, the discharges from the anterior horn cells and the muscular contractions were not due to blood pressure variations. This was demonstrated in experiments in which the blood pressure level was controlled by means of a mercury valve. As can be seen in Fig. 8, injections of pentylenetetrazol and camphor under these conditions produced no variations in blood pressure but caused a series of contractions of the quadriceps muscles, the effect being greatest on the side of the semisection.

2. Experiments in Spinal Cats with Preceding Frontal Lobectomy or Semidecerebration

In the present experiments it was shown that section of spinal connections resulted in an exaggerated sensitivity of the isolated spinal centers to convulsant agents. On the other hand, Magoun and his co-workers (25, 26, 23, 35) have demonstrated that spasticity following cerebral ablations is largely dependent on facilitatory bulbar adaptions. In view of this, it seemed important to ascertain whether the exaggerated responses of the chronic

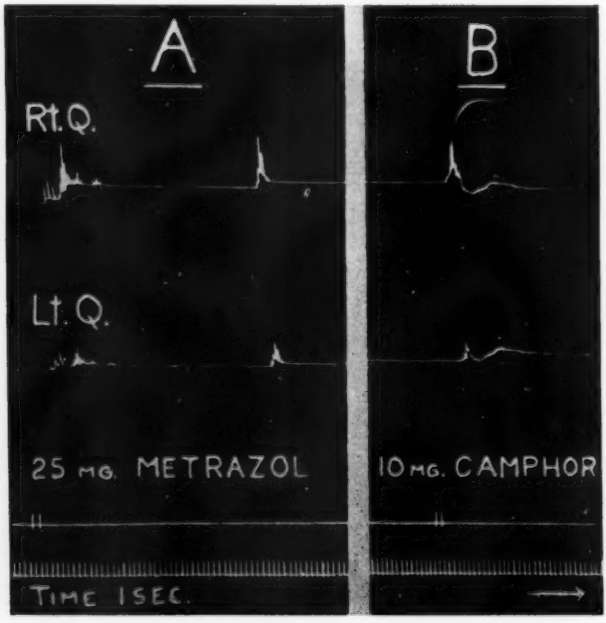

FIG. 9. Effects of pentylenetetrazol and camphor in a cat (2.1 kgm.) 16 days after left semidecerebration. Note practically identical responses on the two sides.

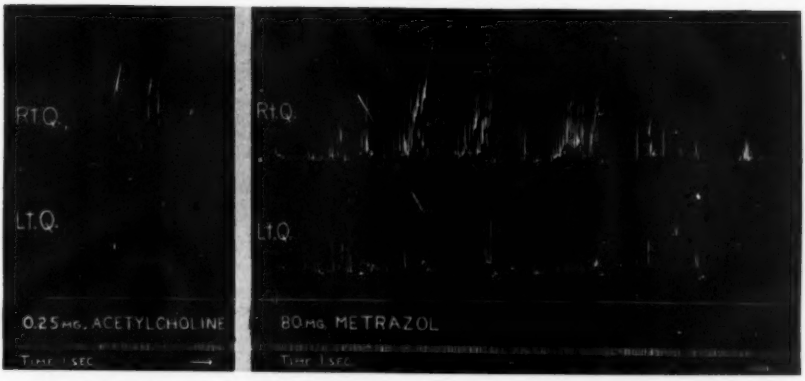

FIG. 10. Effects of acetylcholine and pentylenetetrazol in a cat (2.6 kgm.) three months after left semidecerebration. Note marked exaggeration of the responses on the right side.

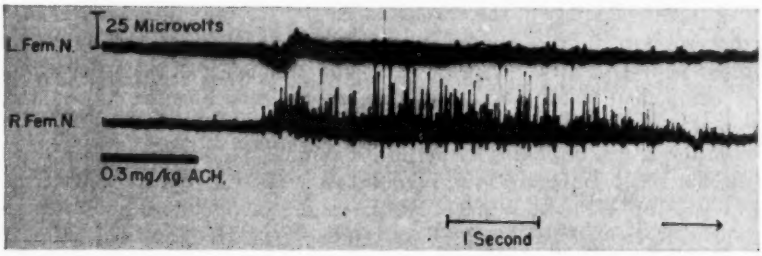

FIG. 11. Effects of intra-aortal injection of acetylcholine recorded from the central ends of severed femoral nerves in a curarized high spinal cat 15 months after left frontal lobectomy. Note lowered threshold on the right side.

frontal lobectomized and semidecerebrated cats depended on a similar mechanism or whether, following cerebral ablation, a degree of supersensitivity to chemical convulsant agents developed in the spinal neurons as well. Nine experiments were done in which the left frontal lobe or a cerebral hemisphere was removed and the cats allowed to live for from seven days to 15 months before they were made spinal and the actual experiments were carried out. As shown in Figs. 9, 10, and 11, the results of intra-arterial injections of acetylcholine, camphor, and pentylenetetrazol in these experiments were not unlike those in which spinal semisection was carried out. Namely, the side of the spinal cord which corresponded to the cerebral ablation was more sensitive to the tested agents than the control side, this being true for strychnine and picrotoxin as well as for the other drugs. However, in these experiments, the supersensitivity of the denervated neurons developed as a rule considerably later than in the case of semisection of the spinal cord and possibly was not as great. Thus in the experiment represented in Fig. 9, it can be seen that 16 days after semidecerebration, the responses to pentylenetetrazol and to camphor were quite symmetrical and only slightly greater on

the sensitized side. However, in an experiment done 120 days after semidecerebration (Fig. 10) a typical asymmetrical response developed when acetylcholine or pentylenetetrazol was injected, the threshold being lower and the latent period being shorter on the side isolated by the semidecerebration. Similarly, in the case of an electrical recording from the femoral nerves (Fig. 11) the spinal neurons which were decentralized by a frontal lobectomy of 15 months' standing responded to smaller quantities of acetylcholine than the ones on the control side.

Discussion

The outstanding finding in the present study seems to be the fact that the median convulsant dose of pentylenetetrazol was reduced in chronic experiments by the preceding removal of one frontal lobe or of a complete cerebral hemisphere. This supports unequivocally the contention of Sauerbruch (34) and of Dandy and Elman (8, 9) who were inclined to believe that cerebral lesions and ablations intensify chemically induced convulsions.

The statistical study of the various aspects of the convulsive patterns lends further support to this conclusion. With an increase in the quantity of injected pentylenetetrazol, the severity and frequency of occurrence of the tonic component of the convulsion were increased both in the control and in the operated animals. The tonic phase was more prevalent at each dose level in the latter, beginning earlier in the convulsion, and lasting longer than in the controls. In regard to intact animals, similar observations were made previously by Pollock, Finkelman, Sherman, and Steinberg (32), while Goodwin, Kerr, and Lawson (17) and Toman, Swinyard, and Goodman (43) associated the tonic extensor component of the seizure with maximal stimulation of the brain.

The terminal clonus of chemically-induced convulsions is often regarded as an interrupted tonic phase (Cobb (5), Strauss and Landis (38), Strauss, Landis, and Hunt (39)). This phase also was lengthened in duration in the operated cats and this may be considered as another manifestation of the greater severity of the seizures in the operated group of animals as compared to the controls.

The latent period between the injection of the convulsant drug and the seizure was less in the operated animals than in the controls, while the mean duration of the convulsions was significantly longer in the operated group. These findings again fit in with the concept that the operated animals were more susceptible to convulsant agents than the controls.

An analysis in photographic recordings of the rolling away from the side of the lesion which occurred in cats with unilateral cerebral ablations during pentylenetetrazol or camphor-induced convulsions showed that the rolling took place during the beginning of the seizure. It was associated with a tonic flexion and withdrawal of the front leg contralaterally to the cerebral ablation and occurred concurrently with clonic extensor thrusts of the limbs on the side of the operation. These extensor thrusts seemed to push the animal

away from the side of the operation, the cat falling and continuing to roll to the side which lost its support due to a tonic flexion of the extremities. Occasionally, very small doses of camphor or pentylenetetrazol caused unilateral extensor thrusts to appear in the front limb opposite to the cerebral ablation in the absence of any convulsive manifestations on the other side of the body. In these instances, the animals fell to the side of the operation. Thus the direction of the rolling and falling of the animals with cerebral ablations, which were previously noted by Pike *et al.* (30, 31) and by Dandy and Elman (8, 9), can be attributed to the exaggerated responses of the denervated side to convulsant agents. The circling of the operated animals which was occasionally seen either after very small quantities of the injected drugs or preceding or following a convulsion may be also explained in this way.

It is of interest to note that the asymmetry during pentylenetetrazol or camphor-induced convulsions in the operated animals was always more pronounced in the forelimbs than in the hind limbs. This fits in with the findings of Lassek (22) who demonstrated that in the cat the majority of the pyramidal fibers terminate in the cervical segments of the cord. An alternative explanation of the greater asymmetry of responses of the forelimbs possibly may be found in the fact that the labyrinthine and neck reflexes exert a synergistic effect on the upper extremity but oppose each other in the lower limb (Magnus and de Kleijn (24)).

In addition to an exaggerated effect of pentylenetetrazol and camphor in chronic frontal lobectomized and semidecerebrated cats, the increased sensitivity of the partially isolated side of the spinal cord to various convulsant agents was demonstrated in sacrifice experiments. Any possibility that the augmented effect of the stimulating agent was due to a penultimate sensitization of the muscles on the isolated side to nerve impulses which originated in the spinal centers was excluded in control experiments in which the discharges of the anterior horn cells were recorded electrically from severed femoral or sciatic nerves. These experiments were considered necessary because of the fact that Cannon and Haimovici (3) described an augmentation of the responses of the denervated quadriceps muscle to acetylcholine on the side of a preceding semisection of the cord.

On the other hand, the augmented sensitivity of the corresponding spinal centers seen in sacrifice experiments after chronic frontal lobectomy or semidecerebration eliminated any possibility that the asymmetrical responses were due to an uneven recovery of the spinal motoneurons from the shock of transection of the cord. Such a condition may have been envisaged in the experiments of Cannon and Haimovici (3). Namely, when at the beginning of a sacrifice experiment the cord was transected below the medulla the preceding semisection located between the transection and the spinal centers of the lower extremity could have protected these centers on one side from the "shock" caused by the transection. In the case of the removal of a frontal lobe or of the complete cerebral hemisphere the chronic lesion was above the site of acute transection thus eliminating this possibility.

Sensitization of the spinal neurons following a frontal lobectomy or a semidecerebration to chemical agents, though definite, seemed to be less pronounced and developed more slowly than after a semisection of the spinal cord, this may be ascribed to the fact that vestibulo- and reticulo-spinal connections remained intact in the case of a frontal lobectomy or semidecerebration but were severed when the spinal cord was semisected. This latter finding is interesting also in view of the fact that Lindsley, Schreiner, and Magoun (23) and Schreiner, Lindsley, and Magoun (35) found that spinal transection abolished the spasticity brought about by cerebral ablations. The difference between the present finding and those of Magoun and his co-workers depends probably on the fact that chemical stimulation brings out the basic sensitivity of the partially denervated neurons more effectively than reflex excitation relied upon in the conditions of the latter experiments; also the time interval allowed for the sensitization to take place in the present study was probably longer than in the afore-mentioned investigation.

It is known that epileptic patients are more susceptible to chemical convulsant agents than normal human beings; this is particularly well substantiated in relation to pentylenetetrazol (Walker (45), Kalinowsky and Kennedy (21), Toman and Goodman (42), von Meduna (27), Kalinowsky (20), Sal y Rosas (33), Goldstein and Weinberg (16), Ziskind and Bercel (47)). Similarly, Pacella, Kopeloff, and Kopeloff (29) and Chusid, Kopeloff, and Kopeloff (4) found that smaller quantities of pentylenetetrazol produced convulsions in monkeys which had epileptogenic lesions induced by the application of alumina cream to the cerebral cortex, while Cure, Rasmussen, and Jasper (7) using a similar procedure showed that subconvulsant doses of pentylenetetrazol activate first the epileptogenic lesion and that electrically recorded epileptic discharges spread from this cortical focus to other parts of the brain. This mechanism they observed also in epileptic patients in whom small quantities of pentylenetetrazol were slowly injected into a vein.

From these findings it is possible to infer that a pathological process of a focal nature or even a widespread derangement of the brain results in an atrophy of short axon neurons in these regions and thus renders other neurons supersensitive to chemical agents or to nerve impulses reaching them by way of the remaining connections, while destruction of long path neurons which results in a degeneration of association, commissural, and descending fibers will sensitize more remote regions of the central nervous system. Evidence that such an interpretation is not too far fetched may be derived from the recent experiments of Echlin and McDonald (11) who isolated neuronally, by a subpial division and undercutting, blocks of cerebral cortex in monkeys and in a schizophrenic patient and found that seven to nine months later the isolated cortex became supersensitive to acetylcholine and pentylenetetrazol and to electrical stimulation as judged by the electrical activity of the isolated regions. Similar results in regard to motor activity were obtained by Obrador (28).

Conclusion

All these considerations lead to the conclusion that partially isolated regions of the central nervous system become 'supersensitive' to convulsant agents such as pentylenetetrazol and camphor. The increase in excitability comes on gradually, and when fully developed, results in a general lowering of the threshold of the animals to chemically-induced convulsions. The change in pattern of the convulsions seen after a unilateral removal of the motor cortex or in frontal-lobectomized or semidecerebrated cats can be accounted for on the same basis.

Acknowledgment

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LIPOTROPIC DOSE-RESPONSE STUDIES IN RATS: COMPARISONS OF CHOLINE, BETAINE, AND METHIONINE[1]

By R. J. Young, C. C. Lucas, Jean M. Patterson, and C. H. Best

Abstract

Dose–response curves have been obtained showing the effects, in weanling rats, of graded doses of choline, betaine, and methionine. respectively, when added to a hypolipotropic diet. A comparison of the lipotropic activity made on the steep portions of the dose–response curves showed that at all liver fat values from normal to 27%, the ratio of betaine to choline necessary to produce a given level of fat was 3 : 1. Essentially similar ratios were observed in the prevention of hemorrhagic kidney lesions and in the stimulation of growth. Methionine, at the lower doses, was as effective as betaine, on a molar basis, in maintaining a given level of liver fat. However, the higher levels of methionine were not as efficient. Methionine at the highest dosage used did not bring the liver fat entirely within the normal range.

Introduction

Some years ago a comparison of the potency of the lipotropic agents was made by Best, Lucas, Ridout, and Patterson (4). The basal diet fed to their rats is now known to have been deficient in the newer B vitamins. Moreover, the protein mixture adopted was purposely designed so that the rats would merely maintain their weight. This was done because Griffith and his co-workers (6, 8) and Beveridge, Lucas, and O'Grady (5) had noted that factors which affect appetite and rate of growth influence the choline requirement of an animal. The effect of calorie intake on the requirement was noted by Best *et al.* (2). Since the results of Best *et al.* (4) cannot be applied to growing animals, we have conducted comparative studies of the lipotropic potency of choline, betaine, and methionine with young rats fed several basal hypolipotropic diets which support optimal growth when adequately supplemented with methionine and choline. After considerable study one of these has been adopted for further studies in this field.

Experimental

White male rats (Wistar strain, weighing from 70–90 gm.) were kept in individual cages with floors of coarse wire screen. Fresh food and water were offered ad libitum and unless otherwise stated, the rats were fed the diets for 21 days. Individual rats were weighed twice weekly and their food consumption was recorded daily. At the end of the experiment surviving animals were anesthetized with ether and decapitated. The livers were removed immediately, wiped free from blood, and weighed. The extraction of the liver lipids with hot alcohol and their rectification with a 3 : 1 (v/v) mixture of petroleum ether and chloroform have been described (3). The kidneys were examined in the gross and the incidence of hemorrhagic lesions was recorded.

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The nature and amount of protein used in the basal hypolipotropic diet was determined in preliminary experiments. On the basis of previous experience by one of us (C.C.L.) with a number of different hypolipotropic diets that had received varying amounts of study, a dietary mixture (Basal A, Table I) was adopted to which equal increments of alcohol-extracted peanut meal (50% protein) and "Alpha" (soya) protein were added at the expense of sucrose.

TABLE I

PERCENTAGE COMPOSITION OF THE BASAL DIETS

	Basal A	Basal B
Alcohol-extracted peanut meal*	8	12
Washed soya protein†	4	8
Casein (vitamin-free)	1	1
L(+)Histidine HCl	0.14	—
L(+)Lysine HCl	0.3	—
DL-Threonine	0.1	—
DL-Methionine	0.4	—
L(−) Cystine	0.2	0.2
Salt mixture‡	3.0	3.0
Celluflour	1.0	1.0
Corn starch	10.0	10.0
Dextrin	10.0	10.0
Sucrose	45.84	38.8
Hydrogenated fat (Primex)	10.0	10.0
Corn oil	5.0	5.0
Sucrose-vitamin mixture‡	1.0	1.0
Cod liver oil concentrate‡	0.01	0.01
d-α-Tocopheryl acetate	0.01	0.01

**Solvent-process peanut meal extracted with hot ethanol (50%, 70%, and 90%).*
†Glidden's "Alpha Protein" washed three times in cold water.
‡Ridout, J. H., Lucas, C. C., Patterson, J. M., and Best, C. H. Biochem. J. 58 : 297. 1954. Vitamin B_{12} (3 mgm. per kgm.) was added to the vitamin mixture described.

This gave a series of test diets of increasing protein content (Table II) which enabled us to determine the protein requirement of young male rats fed this type of diet. The amino acids histidine, lysine, threonine, methionine, and cystine were included in Basal Diet A (Table I) in amounts necessary to bring the essential amino acids of the diet containing 15% protein up to the levels suggested by Rose and his co-workers (9). Weanling rats were fed these test diets for 20 days. Optimal growth occurred with the mixture containing 15% protein (Table II) supplied as 12% extracted peanut meal, 8% soya protein, and 1% casein.

By calculation, the amounts of lysine and threonine in the unsupplemented 15% protein mixture (Basal B) appeared to be borderline. This mixture of dietary protein was therefore tested to see whether it might be deficient in any essential amino acids other than methionine. Growth rates were observed in rats fed Diet B (Table I) plus 0.4% methionine and 0.3% choline Cl, and in similar diets supplemented (*a*) with 0.3% L(+)lysine HCl alone, and (*b*) with 0.3% L(+)lysine HCl plus 0.1% DL-threonine. No differences in rate

TABLE II

EFFECT OF DIETARY PROTEIN LEVEL ON RATE OF GAIN

Diet No.	Supplements to Basal "A"		Total protein, %	Average daily gain (7th to 20th days), gm.
	Peanut meal,* %	Alpha soya protein,* %		
A0	0	0	9	2.3
A1	1	1	10.5	3.5
A2	2	2	12	3.9
A3	3	3	13.5	4.8
A4	4	4	15	5.4
A5	6	6	18	5.5

**Alcohol-extracted peanut meal and water-washed "Alpha Protein" were used.*

of gain or in feed efficiency were observed. These results suggest that the mixture of proteins used in the hypolipotropic diet (Basal B, Table I) is adequate for the rat, since this ration, when supplemented with the lipotropic factors, produced good growth. This diet is much lower in methionine than was that fed by Best *et al.* (4) (190 mgm. versus 360 mgm. per 100 gm. of diet), but is more adequate in all other respects. It was adopted for the present study. Varying levels of choline, betaine, and methionine were added to this basal diet at the expense of sucrose, as shown in Tables III and V.

Results and Discussion

The effects of supplementary choline and betaine are summarized in Fig. 1 and in Table III. The addition of small amounts of choline chloride to the diet gave a dramatic reduction in liver lipids. The amount of choline found necessary for protection against hemorrhagic kidney lesions in young male rats consuming this type of diet was 0.04%, that for maximum growth was 0.10%, and for maintenance of normal liver fat was 0.12% to 0.16%. The corresponding values for betaine under these experimental conditions were

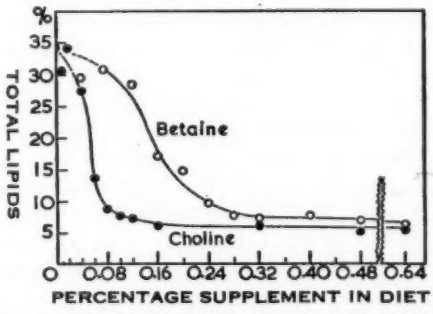

FIG. 1. Dose–response curves showing the effect on total liver lipids (expressed as per cent of fresh weight) of varying amounts of choline (●) and betaine (○) when fed to rats for 21 days.

TABLE III

EFFECT OF CHOLINE AND BETAINE ON FOOD INTAKE, GROWTH, KIDNEY LESIONS, AND LIVER LIPIDS*

Expt. No.	Supplements, %	Survivors / Started	Kidney lesion,† %	Total liver lipids, % wet wt.	Mean values for survivors: Daily food intake, gm.	Mean values for survivors: Daily gain, gm.
	Choline‡					
1	0	2/10	100	34.5 ± 1.80§	10.4	2.52 ± 0.09§
2	0.01	5/11	82	30.9 ± 3.30	9.9	2.61 ± 0.59
1	0.02	7/10	40	34.3 ± 1.02	11.7	2.86 ± 0.31
1	0.04	9/10	0	27.6 ± 2.92	12.9	3.71 ± 0.20
2	0.06	11/11	0	13.7 ± 1.06	11.8	3.84 ± 0.21
1 2	0.08	21/21	0	8.9 ± 0.59	12.0	3.70 ± 0.21
1 2	0.10	21/21	0	7.8 ± 0.18	12.7	4.36 ± 0.25
2	0.12	10/11	0	7.3 ± 0.38	12.8	4.08 ± 0.41
1 2	0.16	21/21	0	6.2 ± 0.12	13.2	4.04 ± 0.15
1	0.32	10/10	0	6.1 ± 0.27	12.4	3.36 ± 0.31
2	0.48	10/10	0	5.2 ± 0.17	13.4	4.20 ± 0.33
2	0.64	7/10	0	5.5 ± 0.15	13.6	4.06 ± 0.29
	Betaine‡					
1	0.04	3/10	90	29.6 ± 5.90	8.8	1.38 ± 0.38
1 2	0.08	7/21	76	30.9 ± 3.06	10.4	2.63 ± 0.31
2	0.12	7/21	60	28.5 ± 2.80	11.9	3.62 ± 0.34
1 2	0.16	19/21	24	17.1 ± 1.64	12.5	3.60 ± 0.15
2	0.20	11/11	9	14.9 ± 1.40	11.9	3.67 ± 0.32
2	0.24	11/11	0	9.8 ± 0.60	11.9	3.82 ± 0.26
2	0.28	11/11	0	7.8 ± 2.80	12.6	3.95 ± 0.39
1 2	0.32	21/21	0	7.5 ± 0.49	13.1	3.94 ± 0.16
2	0.40	11/11	0	7.8 ± 0.69	12.6	3.93 ± 0.28
2	0.48	10/11	0	7.0 ± 0.62	11.9	3.89 ± 0.39
1	0.64	10/10	0	6.2 ± 0.53	14.0	3.98 ± 0.30

Young male rats (Wistar strain), 70–90 gm.
†Incidence of hemorrhagic kidney lesions.
‡Choline was supplied as choline Cl in amounts 1.15 times the value shown. Betaine was supplied as betaine HCl in amounts 1.31 times the value shown.
§Mean ± standard error.

0.20% to 0.24% for protection against hemorrhagic kidneys, 0.24% to 0.28% for optimal growth, and 0.32% for maintenance of normal liver fat. Comparing the amounts of betaine and of choline to maintain any given amount of fat in the liver, we find the average ratio of betaine to choline is about 3.0 : 1 at all liver fat values from normal to 27% (Table IV). Since the molecular weights of the lipotropic compounds are not equal, comparisons should be made on a molar basis; the data have therefore been expressed on both a weight basis and a molar basis. The molecular weights of betaine and choline being nearly equal (117 and 121 respectively) the ratios were essentially the same (3.06 : 1 vs. 3.15 : 1, Table IV). This average ratio is in line with the observation of Griffith and Mulford (7) that under their conditions betaine is approximately one-third as effective as choline. It should be emphasized that such comparisons are valid only when made on the steep portions of the dose–response curves. At higher dosage levels, where both

TABLE IV

LIPOTROPIC RATIO OF BETAINE TO CHOLINE AT DIFFERENT LEVELS OF LIVER FAT

Betaine per 100 gm. diet		Liver fat, %	Choline to give same liver fat		Betaine to choline	
Mgm.	Millimoles		Mgm.	Millimoles	Weight ratio	Molar ratio
120	1.02	26.8	41.7	0.34	2.88	2.97
160	1.37	18.0	54	0.46	2.96	3.05
200	1.71	12.8	62	0.51	3.22	3.33
240	2.05	9.8	74	0.61	3.24	3.36
280	2.39	8.2	92	0.76	3.04	3.12
320	2.73	7.5	105	0.87	3.04	3.12
360	3.07	7.3	110	0.91	(3.27)*	(3.38)*
400	3.42	7.2	118	0.98	(3.39)*	(3.69)*
480	4.10	7.0	120	0.99	(4.00)*	(4.13)*
				Average ratio	3.06	3.15

**Values shown in parenthesis are not included in the average since they were derived from data obtained in the region of maximal response, and not on the steep portion of the dose–response curve.*

TABLE V

EFFECTS OF DIETARY METHIONINE ON FOOD INTAKE, GROWTH, KIDNEY LESIONS, AND LIVER LIPIDS IN THE ABSENCE OF CHOLINE

Supplements*	Survivors / Started	Incidence of kidney lesions	Total liver lipids, % wet wt.	Mean values for survivors: Daily food intake, gm.	Mean values for survivors: Daily gain, gm.
% Methionine in absence of added cystine					
0	7/8	4/8	28.2 ± 2.55†	11.5	1.86 ± 0.20†
0.08	6/8	4/8	28.8 ± 2.91	11.7	2.90 ± 0.22
0.16	8/8	0	21.2 ± 1.68	11.9	3.22 ± 0.21
0.24	8/8	0	13.8 ± 1.91	12.9	3.50 ± 0.30
0.28	8/8	0	13.3 ± 0.74	12.9	3.85 ± 0.27
0.32	8/8	0	12.1 ± 0.92	13.4	3.84 ± 0.33
0.40	8/8	0	11.8 ± 0.88	14.3	4.29 ± 0.29
0.48	6/8	0	9.8 ± 0.51	13.7	3.87 ± 0.28
Methionine with 0.2% cystine added to basal diet					
0	3/8	8/8	31.0 ± 5.08	9.6	1.87 ± 0.29
0.08	5/8	3/8	34.1 ± 0.58	11.3	2.77 ± 0.59
0.16	7/8	1/8	18.8 ± 2.76	12.9	3.56 ± 0.24
0.24	5/8‡	0	11.9 ± 2.03	11.6	3.61 ± 0.39
0.28	7/8‡	0	11.8 ± 1.25	12.3	3.55 ± 0.28
0.32	8/8	0	9.8 ± 0.75	12.9	3.55 ± 0.22
0.40	8/8	0	8.1 ± 0.43	11.6	3.13 ± 0.32
0.48	7/8‡	0	8.3 ± 0.68	11.9	3.28 ± 0.24

**Total methionine content of the basal diet 0.19%, cystine content 0.12%.*
†*Mean ± standard error.*
‡*Mortality due to pneumonia.*

choline and betaine are in excess of the amounts necessary to produce a maximal response, these two substances appear to be about equally effective.

Best *et al.* (4) obtained considerably different results: they observed a wide range of ratios of betaine to choline (varying from 3 : 1 to 8 : 1). Their data were obtained under conditions where growth was purposely kept to a minimum by means of an imbalance of essential amino acids. In addition the diet was deficient in folic acid and in vitamin B_{12} which were not then available. The difference in the findings emphasizes the importance of the nature of the basal diet in such studies.

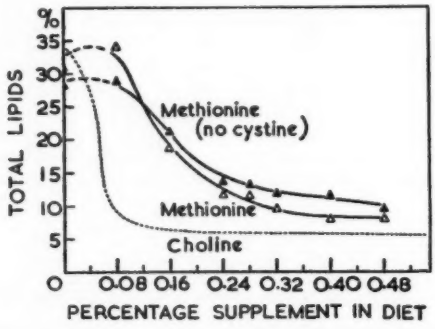

FIG. 2. Dose–response curves showing the effect on total liver lipids (expressed as per cent wet weight) of varying amounts of methionine when fed to rats with (△) and without (▲) supplementary cystine (0.2%).

The difficulties attendant upon attempts to measure the lipotropic activity of methionine have been discussed elsewhere (1, 5, 10–13). The results of another endeavor in this direction using Basal Diet B (with and without added cystine) are summarized in Table V and Fig. 2. Under these conditions methionine was not as effective as betaine, when compared on a weight basis, in preventing the accumulation of fat in the liver, either in the presence or absence of cystine. When the comparison was made on a molar basis (Table VI) the potency of methionine was more comparable with that of betaine. However, unlike the case of betaine, the lipotropic potency decreases as the methionine content of the diet increases. This decrease in lipotropic efficiency of methionine is a linear function occurring in both the presence and absence of added cystine. In this experiment the inefficiency of the higher levels of methionine to reduce the liver fat to the normal range was more evident in the absence of dietary cystine (Table V and Fig. 2). However, rats fed the diet containing the higher levels of supplementary methionine in the absence of added cystine ate more ration and gained in weight more rapidly than rats receiving comparable methionine with cystine. This increased food consumption and concomitant gain in weight probably tended to increase the deposition of liver fat. However, in similar unpublished experiments the greater gain was not always observed in the absence of added cystine.

TABLE VI

RATIO OF METHIONINE TO CHOLINE TO PRODUCE VARIOUS AMOUNTS OF LIVER FAT

Added methionine per 100 gm. diet		Liver fat, %	Choline to give same liver fat		Methionine to choline	
Mgm.	Millimoles		Mgm.	Millimoles	Weight ratio	Molar ratio
*(a) No cystine added to basal diet**						
80	0.54	28.8	36	0.30	2.22	1.80
120	0.81	26.5	42	0.35	2.86	2.56
160	1.07	21.2	51	0.42	3.13	2.54
200	1.34	16.7	56	0.46	3.57	2.89
240	1.61	14.0	59.5	0.49	4.04	3.28
280	1.88	12.8	61	0.50	4.59	3.72
320	2.14	12.3	63	0.52	5.08	4.12
360	2.42	11.8	65	0.54	5.53	4.49
400	2.68	11.5	66	0.55	6.08	5.00
*(b) Cystine (0.2%) added to the basal diet**						
80	0.54	34.1	20	0.17	4.00	3.25
120	0.81	28.8	36	0.30	3.34	2.71
160	1.07	19.5	53	0.44	3.02	2.46
200	1.34	14.6	59	0.49	3.39	2.74
240	1.61	11.8	65	0.54	3.69	2.99
280	1.88	10.2	72	0.60	3.88	3.14
320	2.14	9.5	76	0.63	4.22	3.43
360	2.42	9.2	79	0.65	4.55	3.69
400	2.68	8.8	82	0.68	4.88	3.95

**Basal diet contains 190 mgm. methionine and 120 mgm. cystine per 100 gm. of diet.*

In these experiments higher values were obtained for liver fat at the suboptimal levels of choline or betaine than in the experiments reported by Best *et al.* (4). It is believed that the more adequate diet used in the present study permitted greater growth with the result that more of the limited supply of methionine and choline were utilized for growth, leaving less available for lipotropic purposes. In addition, we know now that the diet used by Best *et al.* (4) was deficient in folic acid and vitamin B_{12}. These vitamins have since been shown to be involved in the metabolism of the lipotropic compounds (*vide* 14). The lower choline requirement and the high betaine-to-choline ratios found at that time were probably due to the higher methionine content of the basal diet coupled with poorer growth.

Summary

A hypolipotropic basal diet is described that contains a minimal amount of protein for optimal growth of rats when supplemented with methionine and choline. Dose–response curves are given showing the lipotropic effects of graded supplements of dietary choline, betaine, and methionine.

The average lipotropic ratio of betaine-to-choline over a range in liver fat from normal to 27% is 3.06 : 1 when compared on a weight basis and 3.15 : 1

when compared on a molar basis. The lipotropic ratios (molar) of methionine-to-choline in the absence of cystine increased from 1.8 to 5.0 and in the presence of added cystine varied from 2.5 to 4.0. Methionine was as effective as betaine (molar) only at the lower dosages. At the higher levels methionine was less effective. Even the highest dose failed to maintain the liver fat within the normal range.

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MAINTENANCE OF BODY TEMPERATURE OF RESTRAINED ADRENALECTOMIZED RATS EXPOSED TO COLD: EFFECT OF ADRENAL CORTICAL HORMONES[1]

By P. F. Iampietro, M. J. Fregly, and E. R. Buskirk

Abstract

Experiments were performed to determine the role of the adrenal glands in the maintenance of colonic temperature of rats which were restrained and exposed to air at 5° C. Colonic temperature decreased linearly with time in the cold air; hence, colonic cooling rate (CCR) was adopted as a measure of response to cold. Bilateral adrenalectomy increased CCR. Administration of cortisone acetate, adrenal cortical extract (ACE) or desoxycorticosterone acetate to adrenalectomized rats decreased CCR. Maximal decrease occurred with administration of 1.00 mgm. cortisone/rat/day or 0.25 ml. ACE/rat/day; higher doses of each hormone were less and less effective in returning CCR toward that of sham-operated rats. An adrenalectomized rat could not be rendered normal with respect to CCR no matter what dose of either ACE or cortisone was administered. In contrast, only the highest dose of DOCA administered (4.0 mgm./rat/day) significantly reduced CCR to that of sham-operated rats. The cooling test is similar to the standard growth test in that the response to administration of cortisone and ACE passes through a maximum and then declines with increasing dosage. The lower colonic temperature of adrenalectomized rats in air at 25° C. was returned to that of sham-operated rats when cortisone (1.00 mgm. or more) or ACE (0.25 ml. or more) was administered; however, colonic temperature did not appear either to increase incrementally or to pass through a maximum with increasing doses of either hormone. DOCA had no effect on initial colonic temperature. Untreated adrenalectomized rats rarely survived lowering of colonic temperature to 22.5° C.; hence, the minimum colonic temperature to which adrenalectomized rats can be cooled and subsequently survive is considerably above that for normal rats (LD_{50} 15.3° C.). Administration of any of the above hormones increased survival even in those doses which did not affect CCR.

Introduction

Adrenalectomized rats in air at 25° C. maintain a constant body temperature but at a level which is usually slightly lower than that of normal rats (25, 26). In air at 5° C., however, these rats cannot maintain a constant body temperature and survive for a relatively short period of time (21, 27). Survival time in cold air has been studied extensively, particularly as a method for assessing the biological activity of adrenal cortical hormones (5, 9, 22). While several investigators (2, 18) have studied the effects of administration of crude adrenal preparations on the maintenance of body temperature of adrenalectomized rats subjected to cold air, assessment has not been made of the effectiveness of individual adrenal hormones. Under conditions of restraint and exposure to air at 5° C., colonic temperature of *normal* rats fell linearly with time in the cold and "colonic cooling rates" could be used for quantitative comparisons (8). This method, adapted for adrenalectomized rats, has been used (*a*) to assess the value of certain adrenal cortical hormones in returning both initial colonic temperature (in air at 25° C.) and colonic cooling rate (in

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air at 5° C.) to normal and (*b*) to ascertain whether the cooling procedure could be used as a bio-assay for the hormones studied. A further aim was to determine the effect of body weight on CCR.

Methods

Two separate series of experiments were performed. The object of the first series of experiments was to determine the relationship between body weight and CCR while the second was performed to determine the effect of administration of adrenal cortical hormones on both the initial colonic temperature and the CCR of adrenalectomized rats. The methods common to both series of experiments are described below.

Male rats of the Sprague–Dawley strain were kept in wire cages in a room maintained at $25 \pm 1°$ C. and illuminated from 8.00 a.m. to 6.00 p.m. All rats were allowed Purina Laboratory Chow, tap water, and 0.15 *M* NaCl solution ad libitum until the cooling test took place.

Colonic temperature was measured using an iron–constantan thermocouple which was inserted 5 cm. into the rat's colon. The thermocouples were led off to a potentiometer which continuously recorded colonic temperature.

The rats were restrained prior to cooling by taping each foot with adhesive tape and leaving a free tab 7 mm. by 30 mm. which was tacked to a board (8). Restraint with the dorsal surface of the body upward and with legs fully extended was used to facilitate heat loss and lessen heat production by muscular activity. After restraint for 15 min. at room temperature, the rats were placed in a thermoregulated box maintained at $5 \pm 2°$ C. Each rat was kept in the cold air until its colonic temperature fell to 22.5° C., at which time it was removed from the box, freed, and rewarmed. Since repeated exposures to air at 5° C. have been shown to adapt rats to cold (i.e., reduce cooling rate) (8), each rat in this and succeeding experiments was used in only one test.

Experiment 1. Relationship Between Body Weight and Colonic Cooling Rate

Over 200 rats with body weights between 80 and 360 gm. were used to determine the relationship between body weight and CCR. To provide proper basis for comparison with adrenalectomized rats, they were all sham-operated one week prior to the cooling test. Sham-operation consisted of removal of a piece of fatty tissue from the region of the adrenal using the standard dorsal approach.

Regression analysis of the relationship between body weight and CCR was performed according to the method described by Ezekiel (7).

Experiment 2. Effect of Administration of Adrenal Cortical Hormones on Colonic Cooling Rate of Adrenalectomized Rats

(*a*) *Effect of Adrenalectomy on Colonic Cooling Rate*

Thirty-two male rats ranging in body weight from 190 to 260 gm. were bilaterally adrenalectomized by the standard dorsal approach one week prior

to the cooling test. Thirty-two sham-operated rats of similar body weight were used as controls. A modified "*t*" test was used for comparison of initial colonic temperature and CCR of the two groups (19).

To test whether the rats in this and succeeding series were completely adrenalectomized salt solution was withdrawn as drinking fluid five to seven days after the cooling test. Completely adrenalectomized rats maintained on Purina Laboratory Chow do not survive longer than 15 days without supplementary NaCl (5, 24). Data obtained from eight adrenalectomized rats were discarded because they survived longer than 15 days after removal of salt. The completeness of adrenalectomy was confirmed by autopsy on all rats that died within 15 days.

(b) Effect of Cortisone Acetate Administration on Colonic Cooling Rate of Adrenalectomized Rats

Eighty-seven adrenalectomized rats were injected intraperitoneally with cortisone acetate[2] once daily for two days prior to the cooling test and one hour before they were subjected to cooling on the third day. Graded doses of cortisone ranging from 0.25 to 2.00 mgm./rat/day were used. A control group consisting of 60 sham-operated rats and 27 non-treated adrenalectomized rats was injected with equal volumes of 0.15 *M* NaCl solution.

The effect of cortisone on CCR was assessed by analysis of variance. This method of analysis was also used in subsequent experiments where more than one dose of a hormone was administered.

(c) Effect of Cortisone Acetate Administration on Colonic Cooling Rate of Normal Rats

Eight normal rats, each weighing approximately 325 gm., were injected intraperitoneally with 2.00 mgm. cortisone acetate once daily for two days prior to the test and one hour before they were subjected to cooling on the third day. A similar number of control rats of the same age and weight were treated with 0.15 *M* NaCl solution. Comparison was made using the "*t*" test referred to previously.

(d) Effect of Adrenal Cortical Extract (ACE) on Colonic Cooling Rate of Adrenalectomized Rats

Twenty-seven adrenalectomized rats were injected subcutaneously with adrenal cortical extract[3] once daily for two days prior to the cooling test and intramuscularly one hour before they were subjected to cooling on the third day. Graded doses of ACE (activity equivalent to 10 glycogen units/ml. or 1 mgm. Compound F/ml.) ranging from 0.25 to 1.00 ml./rat/day were administered. Twenty-seven control rats were injected with equal volumes of cottonseed oil.

[2] *Cortone Acetate, Merck. Courtesy Merck & Co., Rahway, N.J.*
[3] *Lipo-adrenal Cortex in cottonseed oil, Upjohn Co., Kalamazoo, Mich.*

(*e*) *Effect of Desoxycorticosterone Acetate (DOCA) on Colonic Cooling Rate of Adrenalectomized Rats*

Thirty-four adrenalectomized rats were injected subcutaneously with DOCA[4] once daily for two days prior to the cooling test and intramuscularly one hour before they were subjected to cooling on the third day. Graded doses ranging from 0.5 to 4.0 mgm./rat/day were administered. Thirty-four control rats were injected with the same volume of sesame oil.

Results

Experiment 1

Over the range of colonic temperatures from 38° to 22.5° C., the relationship between colonic temperature and duration of exposure to cold air was found to be linear for both sham-operated and adrenalectomized rats. Therefore, CCR (degrees C./hr.) was subsequently used as a measure of body cooling.

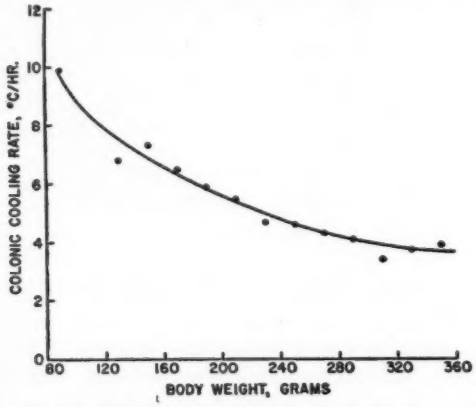

FIG. 1. Relationship between colonic cooling rate in °C./hr. and body weight in grams. Dots represent mean colonic cooling rate for rats in each 20 gm. division. Line is drawn by inspection.

The relationship between body weight and CCR for sham-operated rats is shown in Fig. 1. The least squares equation for the regression line is: log CCR = 2.776 − 0.886 log body weight (standard error of estimate = ± 0.017). Because this relationship existed, body weight had to be taken into account when analysis of variance was used to detect differences in CCR between various treatments. Since a correction involving logarithms is cumbersome, the linearity of regressions of CCR on body weight was tested for the weight range of 190 to 260 gm. which was used in these experiments. Curvilinearity could not be shown for this weight range; therefore, the linear relationship CCR = 7.51 − 0.01 body weight (S.E. = ± 0.0045) was used to correct CCR for the effect of body weight for all rats used in subsequent experiments.

[4]*Percorten Acetate, Ciba Pharm. Co., Summit, N.J.*

It is possible that interactions occur between CCR, body weight, adrenalectomy, and/or hormone treatment. These interactions would alter the assumed independent relationship between CCR and body weight. Additional experiments are planned to clarify this point.

Experiment 2

Bilateral adrenalectomy increased the mean CCR of restrained rats subjected to cold (Fig. 2). The mean rate of cooling of adrenalectomized rats was 7.7° C./hr., while that of sham-operated rats was 4.6° C./hr. ($P < .001$).

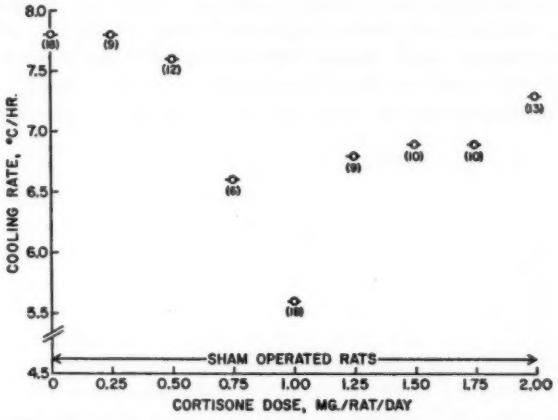

FIG. 2. The effect of various doses of cortisone acetate on colonic cooling rate (CCR) of adrenalectomized rats. The CCR for sham-operated rats is shown in the lower portion of the figure (solid line). Untreated adrenalectomized rats are indicated as 0 dose of cortisone. Number in parentheses refers to number of animals given each dose.

The effect of administration of 0.25, 0.50, 0.75, or 1.00 mgm. of cortisone to adrenalectomized rats was to return CCR toward that of sham-operated rats. The maximal decrease in CCR of adrenalectomized rats was achieved when 1.00 mgm. of cortisone was administered. This dose decreased CCR of adrenalectomized rats from 7.7 to 5.6° C./hr. When doses of cortisone higher than 1.00 mgm. (i.e., 1.25, 1.50, 1.75, 2.00 mgm.) were administered, CCR increased toward that of untreated, adrenalectomized rats (Fig. 2).

Mean colonic temperature of adrenalectomized rats restrained in room air (25° C.) before exposure to cold was consistently lower than that of sham-operated rats (36.7° and 37.7° C. respectively, $P < .01$). Administration of cortisone to adrenalectomized rats in doses higher than 0.75 mgm. returned initial mean colonic temperature to that of sham-operated rats.

Untreated adrenalectomized rats seldom survived lowering of colonic temperature to 22.5° C. Those rats which survived the cooling test died within one day, despite the fact that two methods of artificial rewarming were employed (645 w. infrared heater; immersion to the neck in 38° C. water).

Administration of cortisone in any dose increased survival of adrenalectomized rats; only 12.5% (4 of 32) dying within one day after the cooling test.

It was of interest to determine whether administration of 2.00 mgm. of cortisone/rat/day would also significantly increase CCR of *normal* rats. The results of this experiment indicated that the cortisone treated, normal rats cooled faster than the untreated, normal rats (4.7° and 3.4° C./hr. respectively, $P < .02$). In addition, initial colonic temperatures and body weights were significantly different, cortisone treatment lowered body temperature (38.2° and 37.5° C., $P < .01$) and decreased body weight.

Adrenal Cortical Extract

The effect of administration of 0.25 ml. ACE/day was to decrease significantly the CCR of adrenalectomized rats. However, this rate was significantly different from that of sham-operated rats. Doses of 0.50 and 1.00 ml. of ACE were less effective in reducing CCR (Fig. 3). Hence, the response to administration of ACE was similar to that of cortisone.

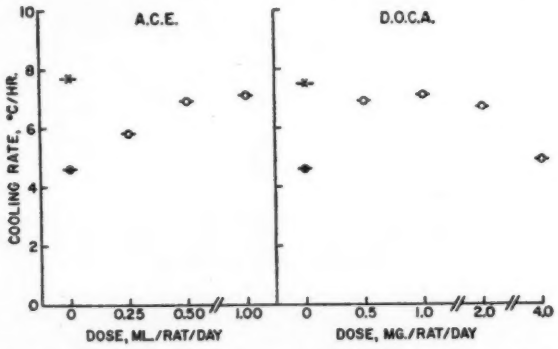

FIG. 3. The effect of various doses of ACE and DOCA on colonic cooling rate (CCR) of adrenalectomized rats. Closed circle represents CCR of sham-operated rats; × represents CCR of untreated adrenalectomized rats.

All doses of ACE were efficacious for survival, none of the treated rats died within one day after the cooling test. Mean colonic temperature of adrenalectomized rats restrained in air at 25° C. was returned toward normal by administration of all doses of ACE.

Desoxycorticosterone Acetate

Administration of 0.5, 1.0, and 2.0 mgm. DOCA/day to adrenalectomized rats was without effect on CCR, while administration of 4.0 mgm. DOCA/day significantly reduced CCR to a level that was not different from that of sham-operated rats (Fig. 3).

All doses of DOCA used were efficacious for survival, none of the treated rats dying within one day after the cooling test. In contrast to the action of cortisone or ACE, all doses of DOCA failed to return to normal the initial colonic temperature of adrenalectomized rats restrained in air at 25° C.

Discussion

The mean colonic temperature of adrenalectomized rats in air at 25° C. is lower than that of normal rats (25, 26). The lower colonic temperature of adrenalectomized rats probably results from their lower metabolic rate, which may be a manifestation of decreased muscular activity. Adrenalectomy is known to reduce the spontaneous running activity of rats (16).

When restrained and exposed to air at 5° C., adrenalectomized rats show an increased rate of fall of colonic temperature. Under these conditions it is obvious that heat loss exceeds heat production. More specific reasons for the faster cooling of adrenalectomized rats are not apparent from these experiments. However, Ring (17) has shown that the heat production of adrenalectomized rats at any given body temperature during cooling was less than that of normal rats while the change of metabolism per change of body temperature was slightly higher. It is likely that the lower metabolism of adrenalectomized rats during cooling is due either to muscular fatigue (less shivering) or to depletion of carbohydrate stores or both. Administration of lower doses of cortisone or ACE to adrenalectomized rats may ameliorate these conditions and thereby reduce CCR. However, it is unlikely that the reversed effect of high doses of cortisone or ACE on CCR seen in this study was to inhibit muscular activity since Ingle (11, 12, 13) showed that administration of adrenal cortical extract, cortisone, or corticosterone to adrenalectomized rats enhanced the work capacity of stimulated muscle in proportion to the amount of hormone administered until an optimal dose was reached. Doses higher than this did not decrease work capacity although none of the single steroid compounds fully substituted for the rat's adrenal cortices. By contrast, Ingle (12) observed that administration of desoxycorticosterone acetate to adrenalectomized rats had little or no effect on work capacity.

The results of the cooling test are similar to those observed in the growth test; viz., the response to administration of cortisone passes through a maximum and then declines with increasing dosage. It has been observed in this laboratory that a dose of 1 mgm./rat/day to adrenalectomized rats maintains a normal growth rate in animals weighing approximately 200 gm. at the time of adrenalectomy. Higher doses tend to reduce rather than increase body weight.

The effect of "higher" doses of cortisone or ACE on CCR observed in this study appear to be similar for normal and adrenalectomized rats because CCR of normal rats was increased when a 2.00 mgm. dose of cortisone was administered. Recent work on normal rats has indicated another possible explanation for these findings. Large doses of cortisone have been shown to inhibit both uptake (1, 15, 23) and release (4) of I^{131} by the thyroid gland. Furthermore, both Bodansky and Money (3) and Essex (6) have observed that administration of 1.5 mgm. of cortisone/rat/day for several weeks resulted in reduction of wet weight of the thyroid gland as compared with controls of the same age. Experiments are being performed to determine the role of the thyroid in the faster cooling which accompanies the administration of high

doses of cortisone. These experiments have been designed to determine if the inhibition resulted from modified thyroid function or modified response of cells to normal thyroid function.

In our hands, ACE gave a pattern of results similar to cortisone. It is difficult to explain these results on the basis of inhibition of thyroid function since clear-cut inhibition of uptake of I^{131} by the thyroid gland of rats was not demonstrated by Pasckis *et al.* (14) when high doses of ACE were given. It is possible, however, that the "inhibitory" effects of administration of large doses of ACE are manifested at the cellular level.

DOCA, in contradistinction to ACE, reduced the CCR of adrenalectomized rats toward that of sham-operated rats only when given in relatively high doses (4.0 mgm./rat/day). The literature on the effect of administration of high doses of DOCA on thyroid function is conflicting, since both inhibition (patients (28)) and no inhibition (rats (14)) have been reported.

An adrenalectomized rat could not be rendered normal with respect to CCR no matter what dose of either ACE or cortisone was administered. Of the hormones used only DOCA (4.0 mgm./day) significantly reduced CCR of adrenalectomized rats to that of sham-operated rats. It has also been observed that the work capacity of adrenalectomized rats could not be returned to that of normal rats by administration of large doses of compounds E, F, or B individually or simultaneously (13). However, administration of ACE to adrenalectomized rats did maintain normal work capacity. With regard to ACE, the results of the cooling rate studies agree more closely with those of Heroux and Hart (10) who reported that growth rates of adrenalectomized rats given graded doses of ACE could not be returned to normal. Survival time of the ACE-treated adrenalectomized rats at $-29°$ C. was also less than that of sham-operated rats. These investigators have suggested that some additional factor not present in commercial extracts may be necessary for restitution of normal function.

Untreated adrenalectomized rats rarely survived lowering of colonic temperature to 22.5° C. They died within one day after the cooling test. Although two methods of rewarming failed to alter survival rate, administration of any of the hormones used enabled the rats to survive cooling and subsequent rewarming. The fact that untreated adrenalectomized rats died after cooling to 22.5° C. is in direct contrast to the response of normal rats, which can be cooled to 18° C. colonic temperature and rewarmed with no apparent ill effects (8). Hence, it would seem that the minimum colonic temperature to which adrenalectomized rats can be cooled and subsequently survive is considerably above that for normal rats. It is also interesting that survival was enhanced by administration of all doses of cortisone, ACE, or DOCA even in those that did not affect CCR.

The results reported here for adrenalectomized rats confirm the findings of various authors. Adrenalectomized rats manifest a lower colonic temperature in air at 25° C. (26), an increased rate of fall in colonic temperature on exposure to cold (9, 20), as well as a decrease in survival time (5, 21, 22).

Administration of some compounds possessing adrenal cortical activity tends to maintain colonic temperature of rats in air at 25° C., to partially maintain colonic temperature in cold air, and to improve survival.

Both Baird *et al.* (2) and Roos (18) suggested that the fall in colonic temperature on exposure to cold might be used as a quantitative bio-assay. The dose–response relationship demonstrated here clearly indicates that neither CCR nor initial colonic temperature measurements can be recommended as a convenient bio-assay for the hormones studied.

Acknowledgments

It is a pleasure to acknowledge the valuable counsel of Dr. E. Knobil and the technical assistance of Miss Cynthia S. Cook. We thank Mrs. J. Swan of the Statistical Office of the QM Research and Development Command for the statistical analyses, and Dr. A. Henschel for helpful comments.

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THE OCCURRENCE OF ACETYLCHOLINE IN THE HOUSEFLY, MUSCA DOMESTICA L.[1]

By W. Chefurka and B. N. Smallman

Abstract

The occurrence of acetylcholine in the heads of houseflies has been demonstrated. After a preliminary purification by paper electrophoresis, acetylcholine was identified by paper chromatography, paper electrophoresis, pharmacological methods, and by its reaction with hydroxylamine – ferric chloride. The base was also isolated as a reineckate, converted to the perchlorate, and its infrared spectrum shown to be identical with reagent acetylcholine.

Introduction

Insect nervous tissue exhibits high cholinesterase activity (see 20). If this enzymic activity is inhibited, normal synaptic function is disturbed (19, 21). By analogy with vertebrates, this disturbance may be due to an accumulation of Ach.[2] However, synaptic conduction in the roach is completely unaffected by exogenous Ach or other choline esters (19), and the suggestion has also been made that Ach may not be present in insects (18). It seems important therefore, to establish unequivocally whether Ach is a component of insect nervous tissue.

The evidence of the early investigators for the presence of Ach in insects is inconclusive because they employed either the frog's rectus abdominis or the leech muscle. As pointed out by Chang and Gaddum (4), such evidence by itself is inadequate and indicates only an Ach-like substance.

Recent investigations have strongly suggested that insects do contain Ach. Lewis (13), on the strength of combined chromatographic and pharmacological evidence, has suggested that the active principle in *Calliphora* is Ach. Augustinsson and Grahn (2) have prepared reineckate and tetraphenylboron complexes with nitrogenous bases from bee heads, and found by chemical and biological methods, Ach along with two unidentified compounds.

The present communication deals with the identification of Ach in the housefly head. By means of paper chromatography, paper electrophoresis, pharmacological bio-assays, and infrared spectrophotometry, it has been established that the Ach-like substance extracted from the heads of houseflies is identical with Ach itself.

A preliminary report of this work has appeared elsewhere (6).

Materials and Methods

Insects

The insects used in this study were adult houseflies, *Musca domestica* L., one to four days old. They were taken from a laboratory culture maintained according to Fisher (9).

[1]*Manuscript received February 24, 1956.*
Contribution No. 70 from Canada Department of Agriculture, Science Service Laboratory, London, Ontario.

[2]*The following abbreviations are used throughout this paper: Ach, acetylcholine; TCA, trichloroacetic acid; ChE, cholinesterase.*

Chemicals

The chromatographic solvents were of reagent grade. Potassium bismuth iodide was purchased from British Drug Houses, the Reinecke salt from Fisher Scientific Co., and silver perchlorate from Bios Laboratories, Inc. Purified bovine erythrocyte cholinesterase was purchased from General Biochemicals Co. Ach perchlorate was prepared by mixing equimolar amounts of Ach chloride and silver perchlorate; the silver chloride produced in this reaction was collected by centrifugation.

Preparation of Tissue Extracts

Usually a large quantity of flies was frozen on dry ice and decapitated. The fly heads were then stored at $-10°$ C. until ready for use. Between 10 and 15 gm. of frozen fly heads was homogenized in a Waring blendor for five minutes in 100 ml. of 0.01% eserine sulphate. The pH of the homogenate was adjusted to approximately 2 with HCl and allowed to stand at room temperature for 100 min. to release the bound Ach (15). Enough solid TCA was added to make a 5% solution and after 5 to 10 minutes, the precipitated proteins were removed by centrifugation at $3000 \times g$ for 30 min.

The clear brownish supernatant was decanted and the precipitate was washed and centrifuged. The supernatants were pooled and the TCA was removed by extracting three times with an equal volume of ether. The pH of the solution at this point was in the neighborhood of 4. The extract was then reduced to dryness *in vacuo* at a temperature not exceeding 40° C. The brownish residue was extracted five times with 1 ml. portions of ethanol. After the ethanolic extract was cleared by centrifugation, it was reduced to dryness in an air stream. The residue was dissolved in an appropriate volume of distilled water and employed in the various procedures of characterization outlined below.

Results

Methods and results specific to the various characterization procedures are detailed in the appropriate sections below.

(*1*) *Characterization of Ach by Paper Electrophoresis*

Preliminary attempts to chromatograph the tissue extracts failed because of the presence of interfering materials. However, by taking advantage of the strongly basic character of Ach, it was possible to separate the active component from the interfering materials by paper electrophoresis.

The electrophoresis apparatus was manufactured by Shandon Scientific Co. Lithium sulphate of ionic strength 0.10 served as the electrolyte. Whatman No. 3 MM filter paper, cut into strips $15\frac{3}{4}$ in. by 1 in., was dipped in the electrolyte and the excess was blotted. A period of one hour was allowed for equilibration at the appropriate voltage. After electrophoresis, the choline esters were located by spraying the paper strips with potassium bismuth iodide (3), or with phosphomolybdic acid followed by reduction with acidic stannous chloride (5).

In the early experiments, the electrophoresis was carried out at 500 v. for one hour. However, more satisfactory results were obtained at 200 v. for four hours and these conditions were used for most of this work. The brownish extract was applied, as a narrow band, on several filter paper strips with a camel's hair brush. At the end of the run, the filter paper strips were dried at 110° F. in an aerated oven and the papers were sprayed to locate the bands.

The results of a typical experiment are shown in Fig. 2. It is clear that two bands moved to the cathode indicating that these substances were positively charged. Furthermore, most of the brown interfering material remained at the point of origin (0). When the filter paper strip was sprayed with potassium bismuth iodide (Fig. 2A) the first band was bluish-orange, while the second band was yellowish-orange in color. Controls of Ach and choline, when sprayed with potassium bismuth iodide, gave a yellowish-orange and a bluish-orange color, respectively. These findings suggested that the first band was due to choline or a compound of equal basicity, and the second to Ach.

The second band was therefore eluted with ethanol for 40 min. The eluate was reduced to dryness in a stream of air and the residue was dissolved in 0.15 ml. of distilled water; to half of this volume was added 100 μgm. of reagent Ach. Both these solutions were then applied to the filter paper strips along with a control of reagent Ach, and electrophoresed for one hour at 500 v. The filter paper strips were then dried and sprayed with phosphomolybdic acid.

The results presented in Fig. 1 show that when the material eluted from band 2 was mixed with Ach, no resolution of the substances was obtained. Furthermore, the eluate from band 2 moved with a mobility which was identical with that of reagent Ach.

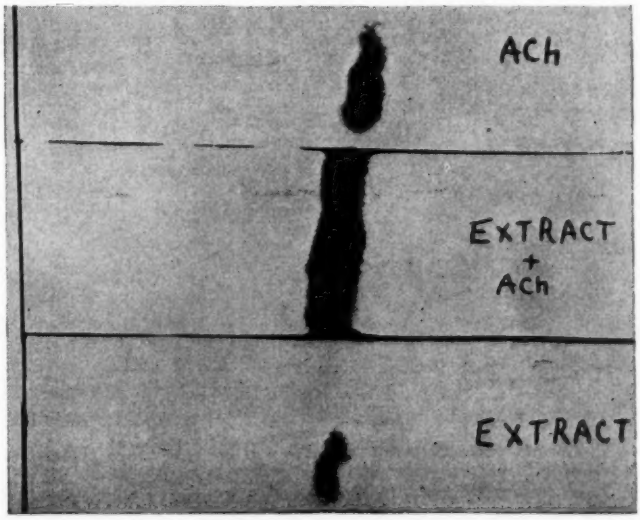

FIG. 1. Paper electrophoresis strips of: extract from band 2, mixture of this extract and reagent Ach, and reagent Ach alone.

(2) Characterization of Ach by Paper Chromatography

Sections of the electrophoresis strips corresponding to the location of bands 1 and 2 were cut out, eluted, and chromatographed.

Paper chromatography was carried out using the ascending method of Williams and Kirby (24). The extracts were spotted on Whatman No. 1 filter paper from micropipettes obtained from Microchemical Specialities Co. For comparison, reagent Ach bromide was chromatographed, as well as Ach bromide which had been run parallel with the extract through the electrophoresis procedure.

The chromatograms were run overnight (approximately 16 hr.) in butanol – acetic acid – water, 4 : 1 : 5 (17). After the chromatograms had been dried in an aerated oven at 110° F., the spots were visualized by spraying with phosphomolybdic acid followed by acidic stannous chloride.

Fig. 3 shows the results. Band 1 (E_1) was resolved as a single band which was probably choline (see section 5). Band 2 (E_2) was resolved into two components. The identity of the fast-moving spot in unknown. The other

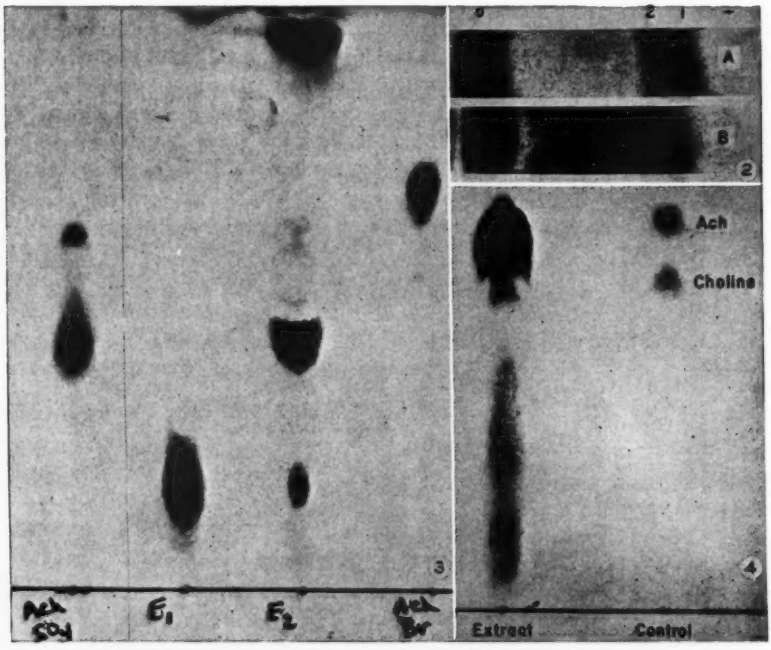

FIG. 2. Paper electrophoresis strips of extracts of housefly heads sprayed with: *A*, potassium bismuth iodide; *B*, phosphomolybdic acid – stannous chloride.

FIG. 3. Paper chromatograms of: eluates from bands 1 and 2 (E_1 and E_2) obtained by electrophoresis of housefly extracts; Ach after electrophoresis (Ach SO_4); Ach Br.

FIG. 4. Paper chromatogram of: housefly ester sample, and a mixture of Ach perchlorate and choline perchlorate.

component had an R_F (0.40) identical with that of Ach sulphate subjected to the same electrophoresis procedure as the extract; the faint lowest spot with R_F identical to band 1 (E_1) probably resulted from incomplete separation of the bands 1 and 2. The R_F of Ach bromide was considerably higher, suggesting that the anion exerts a considerable influence on the R_F of these compounds. Similar findings were reported by Whittaker and Wijesundera (23). It appears reasonable to conclude therefore, that the valid comparison is between the extract and Ach treated in the same manner, and that this comparison indicates the presence of Ach in band 2.

(3) Characterization of Ach by Pharmacological Activity

The frog's rectus abdominis is one of several biological objects sensitive to the low concentrations of Ach. The substance present in band 2, and already indicated to be identical with Ach, was tested for biological activity, using the eserinized frog's rectus abdominis (4). As was found by Augustinsson and Grahn (2), the extraction procedures resulted in an extensive hydrolysis of Ach, but a sufficient amount remained to permit a satisfactory bio-assay.

After a preliminary electrophoresis of the tissue extracts, bands 1 and 2, as well as the remainder of the filter paper strip were eluted in 10^{-4} N HCl in ethanol (hereafter termed acidified ethanol) for 40 min. (23). The eluates were dried in a stream of air, the residue dissolved in amphibian-Ringer's solution and assayed on the eserinized frog's rectus abdominis. Fig. 5 clearly shows that the extract, before electrophoresis, was active. After electrophoresis, most of the activity was localized in band 2; band 1 was inactive, and the remainder of the paper electrophoresis strip showed negligible activity probably resulting from retention of Ach by the thick mass of extract at the origin.

Eluates from sections of a paper chromatogram similar to that shown in Fig. 3, were also assayed pharmacologically. The results showed that all the activity was present in the spot with R_F identical to Ach, and no other portion of the paper chromatogram showed any pharmacological activity.

(4) Chemical Characterization of Ach

Hestrin (11) has shown that Ach may be assayed chemically by reaction with hydroxylamine – ferric chloride to give a yellowish colored complex with an absorption curve having a shoulder at 540 mμ.

Bands 1 and 2, after paper electrophoresis, were eluted in acidified ethanol for 40 min. The eluates were reduced to dryness in a stream of air and the

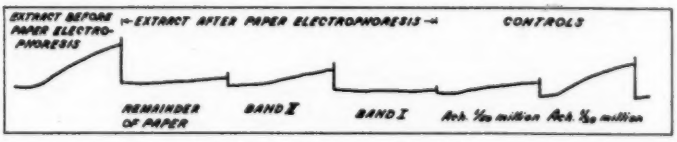

FIG. 5. Response of eserinized frog's rectus abdominis to: extract from housefly heads before paper electrophoresis; eluates from bands 1, 2, and remainder of electrophoresis strip; and to reagent Ach.

residue was dissolved in 1 ml. of distilled water. These solutions were then treated according to Hestrin (11) and the absorption spectra determined using a Beckman spectrophotometer, Model DU.

Fig. 6 shows that the absorption spectrum obtained with the eluate from band 2 was identical with that obtained with reagent Ach; both display a shoulder at 540 mμ. No shoulder at this wave length could be detected for the material eluted from band 1. The optical density of band 2 at 540 mμ was 0.067 which corresponds to 22 μgm. of reagent Ach. Since band 2 was derived from an extract of 14.6 gm. of fly heads, the Ach content was indicated as about 1.5 μgm./gm. heads. Because Lewis, Smallman, and Hallowes (private communication) have established that extracts of fly heads contain 26.1 μgm. Ach/gm. heads, it is clear that approximately 90% of Ach was lost during our extraction procedure. This agrees with the estimates of Augustinsson and Grahn (2) who state that no more than 10–20% of the original activity of bee head extracts was found in the final solution after their fractionation procedure.

(5) *Characterization of Ach by Hydrolysis with Cholinesterase*

Approximately 12 gm. of frozen fly heads was homogenized in 100 ml. distilled water for five minutes. The homogenate was adjusted to pH 2.1 and allowed to stand at room temperature for 100 min. to release all the bound Ach. After the pH was adjusted to 7.40, 10 ml. of purified Ch E(1 mgm./ml.) was added and allowed to react with the homogenate for 25 min. The pH of the homogenate at the end of this period had dropped to 6.94 indicating the release of acid, presumably from the hydrolysis of endogenous Ach. The reaction was stopped with 10% TCA, and the procedure after this point was identical with that outlined under "preparation of tissue extracts".

The extract was electrophoresed at 200 v. for eight hours. The electrophoresis strip was cut into two portions, one of which was sprayed with

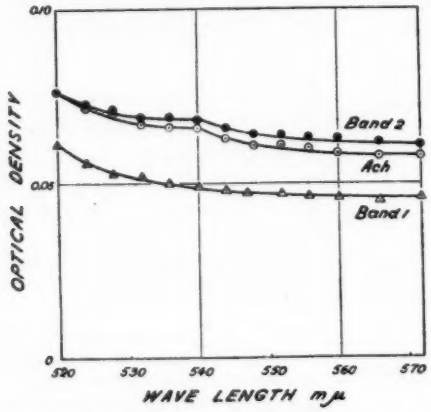

FIG. 6. Absorption spectrum of: eluates from bands 1 and 2, and of reagent Ach reacted with hydroxylamine – ferric chloride.

potassium bismuth iodide. Instead of the usual two bands, only one band appeared. The corresponding area of the other portion of the strip was eluted in acidified ethanol and re-electrophoresed at 200 v. for two hours. Ach and choline were run as controls.

Fig. 7 shows that the substance remaining after treatment of the extract with cholinesterase had the same mobility and gave the same color reaction (bluish-orange) as choline.

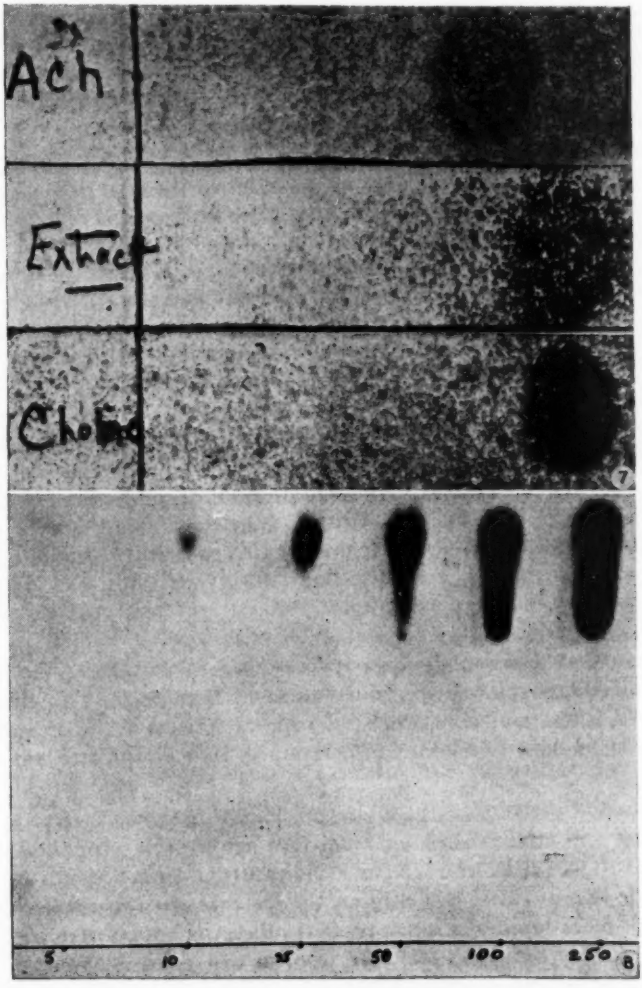

FIG. 7. Electrophoresis strips of: reagent Ach, reagent choline, and extract of housefly heads after treatment with ChE.

FIG. 8. Paper chromatogram showing the effect of amounts of Ach chloride on formation of double spots.

(6) Characterization of Ach by Infrared Spectrophotometry

The Ach molecule is distinguished by the presence of an acetyl and a carbonyl group. Both of these functional groups have a characteristic group vibration frequency which shows up as an absorption band in the infrared region.

Approximately 1 kgm. of frozen adult houseflies was homogenized in a Waring blendor for five minutes in 2 liters of ethanol at 2° C. To this homogenate was added 225 gm. of solid TCA and the mixture was allowed to stand overnight at 2° C. The extract was filtered through cellulose powder and the clear brown solution was reduced *in vacuo* to approximately 200 ml. To this brown viscous liquid was added an equal volume of water and the precipitate which formed was eliminated by centrifugation.

The brownish liquid was extracted three times with an equal volume of ether to remove the TCA. From this point we followed the procedure of Gardiner and Whittaker (10) for the precipitation of tissue bases as reineckate salts. The reineckate precipitate was immediately converted to the perchlorate salt with silver perchlorate. After lyophilization, the solid residue was extracted four times with several milliliters of ethanol and the extract was cleared by centrifugation at 8000 $\times$ *g* for 10 min. To the alcoholic extract was added seven volumes of ether. A white precipitate appeared which, after one hour at 2° C., was centrifuged and dried in a desiccator overnight.

The precipitate turned into a brownish resinous mass overnight. It was again extracted with ethanol, cleared by centrifugation, and treated with one volume of ether to yield a brownish precipitate. Because preliminary experiments showed that Ach perchlorate is not precipitated under these conditions, the brownish precipitate was discarded. To the supernatant was then added six volumes of ether. A fine, white precipitate appeared. After one hour at 2° C., it was collected by centrifugation and divided into several portions which were used for chromatography and infrared spectrophotometry as detailed below.

The infrared studies were carried out with this precipitate using the Perkin–Elmer double beam infrared spectrophotometer. Because the precipitate was hygroscopic, it was mulled between two rock salt prisms in the presence of a few drops of nujol. The infrared spectrum of Ach perchlorate was run as a control.

The infrared spectrum of the white precipitate is shown in Fig. 9. The spectrum of the material isolated from flies agrees well with that of Ach perchlorate. The peak at 1725 cm.$^{-1}$ indicates the presence of a carbonyl group, while that at 1240 cm.$^{-1}$ suggests the presence of an acetyl ester. The weakness of these two peaks is due partly to the interference from the strong water peak and a sodium perchlorate peak in the region 1635 cm.$^{-1}$. The other characteristic peaks occur at 930 cm.$^{-1}$, 870 cm.$^{-1}$, and 838 cm.$^{-1}$. The broad intense peak in the region of 1100 cm.$^{-1}$ is due to the perchlorate ion. The positions of these agree with those given by Wood (25) for Ach perchlorate.

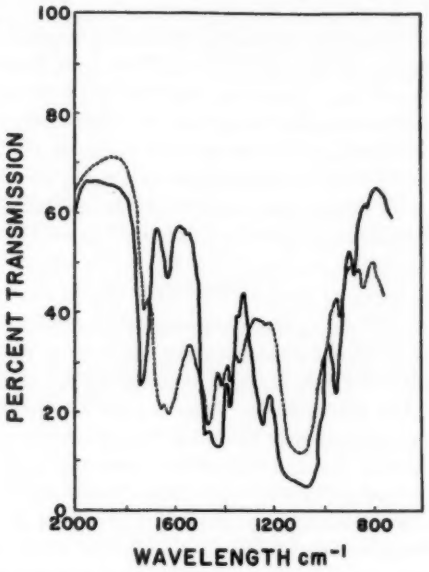

FIG. 9. Infrared spectrum of: housefly ester sample — - —, synthetic Ach perchlorate ————.

The presence of Ach perchlorate in this preparation was also confirmed by chromatography. Fig. 4 clearly indicates the presence of Ach perchlorate (R_F 0.40) as well as some choline perchlorate (R_F 0.34).

(7) Behavior of Ach During Chromatography

During this study we have often observed that under certain conditions choline esters chromatograph as double spots. Augustinsson and Grahn (1) also allude to this phenomenon.

This phenomenon appears to be a function of the amounts of choline esters applied to the paper. Fig. 8 shows that increasing the amount of Ach chloride above 25 μgm. results in an increasing tendency to form double spots. This

TABLE I

THE EFFECT OF AMOUNT OF ACH CHLORIDE, ACH BROMIDE, AND ACH ACETATE ON THE R_F AND NUMBER OF SPOTS

Amount, μgm.	Ach Br		Ach Cl		Ach acetate
	R_F	Remarks	R_F		R_F
10	0.56		0.61		0.57
25	0.58		0.61		0.56
50	0.62	Beginning of tailing	0.61	0.46	0.60
100	0.66	0.55	0.62	0.47	0.62
250	—		0.63	0.51	0.66

phenomenon was displayed by all choline esters tested; that is, by choline chloride, Ach chloride, benzoylcholine chloride, succinylcholine chloride, and propionylcholine chloride. However, we confirmed the suggestion of Munier and Macheboeuf (16) that this formation of double spots may be eliminated when the anion associated with the ester was the same as the anion of the acid used in the solvent. Table I shows that Ach acetate runs as a single spot in butanol – acetic acid – water, in contrast to the double spots formed by Ach bromide and Ach chloride in amounts above 50 μgm.

Discussion

In the present study the substance in extracts of fly heads, which activated the eserinized frog's rectus abdominis, was shown to be identical with Ach. After electrophoresis of crude extracts, this biological activity was found in a band which moved to the cathode; when eluates of this band were chromatographed, biological activity was localized in a spot with an R_F corresponding to Ach. No evidence for the occurrence of other substances with similar biological activity was obtained. If other choline esters are present in fly head extracts or the reineckate precipitates of whole flies, then either they occur in low concentrations or they are different from butyrylcholine, propionylcholine, and benzoylcholine, all of which were found to have R_F's higher than Ach. In contrast to the presence of a single choline ester in the head of the housefly, Augustinsson and Grahn (2) have presented evidence for two unidentified choline esters as well as Ach in the head of the honeybee. Recent evidence suggests that different choline esters may exist in different species (7) or in different tissues within the same species (10).

The identification of Ach in extracts of housefly heads proved difficult because of the presence of large quantities of interfering materials. The presence of these substances may partly account for Fernando's (8) failure to obtain a positive Hestrin test in extracts of housefly heads. We obtained a positive Hestrin test after a preliminary purification of the biologically active extract by paper electrophoresis. Indeed, a degree of purification was also essential to the identification of Ach in our extracts by the methods of paper chromatography and infrared spectrophotometry.

A second difficulty was encountered when we found that Ach in our extracts tended to chromatograph as double spots especially when large amounts of extract were applied. With reagent Ach we also found that the tendency to run as double spots increased with the amount applied. This difficulty was obviated by working in the range from 10–50 μgm. Ach where double spots did not occur. Munier and Macheboeuf (16) also encountered this difficulty in chromatographing a number of nitrogenous bases, and found that double spots or tailing was prevented when the anion associated with the ester was the same as the acid anion used in the solvent. In confirmation, we found that when reagent Ach in our experiments was converted to the acetate, and run in butanol – acetic acid – water, the ester moved as a single spot. Furthermore, we confirmed with Ach the finding of Munier and Macheboeuf (16)

that the R_F of these nitrogenous bases was more constant in an acid than in a neutral solvent, although we still encountered some variation in R_F with different batches of the acidic solvent.

A third difficulty that may interfere with a positive identification of Ach in tissue extracts is the effect of the anion on the R_F. Whittaker and Wijesundera (23) have reported that the R_F of Ach varied with the anion. We also found that the R_F varied from about 0.40 with the perchlorate or the sulphate to about 0.60 with the chloride, bromide, or acetate.

Because of these difficulties, the characterization of Ach in extracts of housefly heads by paper chromatography became possible only when care was taken to treat the reagent Ach, used as a control, in the same manner as the tissue extract; moreover, it was important to apply both the extract and the reagent Ach in quantities low enough to avoid the formation of double spots or tailing. When these precautions were taken, as for instance when reagent Ach was run parallel with the extract through the electrophoresis procedure, or when both reagent Ach and the reineckate salt of the extract were treated with perchlorate, then identical R_F's were obtained for a component in the flies and Ach itself.

The Ach content of housefly heads as estimated in eluates of paper electrophoresis strips by Hestrin's method was about 1.5 μgm./gm. of tissues. This value is considerably lower than the values obtained by others (see 20) for the pharmacologically active substance in insects. Losses of Ach during extraction of the housefly heads probably occurred, as in fact Augustinsson and Grahn (2) found with bee heads. Moreover, in our experiments the heads were frozen and stored immediately after decapitation; subsequent work by one of us (B.N.S.) with other collaborators has shown that after freezing, large losses may occur by enzymic hydrolysis during the extraction of the Ach-like substance from *Calliphora* heads (Lewis, Smallman, and Hallowes, private communication). These workers have examined various insect tissues using methods designed to prevent the hydrolysis and synthesis of Ach during the extraction procedure. With extracts of housefly heads, assayed on the frog's rectus abdominis, they obtained a mean value of 26.1 μgm. Ach/gm. of heads. Previously, values for the Ach content of insect tissues were obtained by the method of pharmacological assay without critical evidence for the identity of the active substance or substances. In this case however, the value obtained for the housefly is supported by our evidence that extracts of *Musca* heads contain only one substance active on the eserinized frog's rectus abdominis, and that this substance is identical with Ach.

The demonstration of Ach in an insect is pertinent to the hypothesis that the organophosphate insecticides kill insects by inhibiting the ChE of the nervous system, and to the corollary assumption that Ach is the synaptic mediator in insects. Hopf (12) and Lord and Potter (14) have objected to this hypothesis partly on the grounds that the evidence for the occurrence of Ach in insects is inadequate. This objection now seems untenable. Our several lines of evidence consistently show that the Ach-like substance in the

housefly is identical with Ach itself; and others have recently published chromatographic evidence for the occurrence of Ach in blowflies (13) and honeybee (2). Further support for these findings has been provided by the recent demonstration of the enzymic mechanisms for the synthesis of Ach in the blowfly (22). It seems probable, therefore, that Ach is a normal component of the nervous tissue of insects.

Our objective in this study has been to establish, as unequivocally as possible, whether Ach occurs in an insect; this seemed the necessary first step in any approach to the problem of the physiological basis for the action of the organophosphate insecticides. With the demonstration of Ach in insects, it becomes possible to take the second step and inquire into its physiological function. Since Ach and the enzymes required for its metabolism are now known to occur in insects, it is clearly a candidate for the role of synaptic mediator in insects, as in vertebrates. However, important differences such as the insensitivity of insects to injected Ach, the much higher content of Ach in insect nervous tissue, as well as the higher activity of the enzymes responsible for its synthesis (22) and hydrolysis (20) may indicate different or additional physiological functions of Ach in insects.

Acknowledgment

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SOME OBSERVATIONS CONCERNING THE BIOCHEMICAL INERTNESS OF METHYLPHOSPHONIC AND ISOPROPYL METHYLPHOSPHONIC ACIDS[1]

By F. C. G. Hoskin

Abstract

Methylphosphonic acid is not degraded in the intact rat to phosphoric acid. The stability of isopropyl methylphosphonic acid in the intact rat has been confirmed. Rat muscle phosphorylase appears unable to catalyze the synthesis of hexose phosphonate esters from methylphosphonic or isopropyl methylphosphonic acids under conditions where hexose phosphate esters are readily synthesized from phosphoric acid.

Introduction

The powerful cholinesterase inhibitor, sarin (isopropyl methylphosphonofluoridate), has been shown to be enzymatically hydrolyzed to monobasic isopropyl methylphosphonic acid (1, 7). In the intact rat, this hydrolysis product was not further hydrolyzed to dibasic methylphosphonic acid (7). Nevertheless, despite the fact that it is oxidized to phosphoric acid under only the most vigorous conditions (6), methylphosphonic acid may be considered to be the penultimate chemical degradation product of sarin.

The present experiments were undertaken to illustrate the fate of methylphosphonic acid in the intact animal. In addition, because of the chemical similarities between phosphoric and methylphosphonic acids (6), the possibility of the biosynthesis of phosphonate esters analogous to the hexose phosphates was investigated. The biochemical stability of isopropyl methylphosphonic acid (7) has also been confirmed.

Materials and Methods

P^{32}-Methylphosphonic acid was synthesized by the spontaneous hydrolysis of P^{32}-methylphosphonyl dichloride* in an excess of distilled water. The solution was evaporated to dryness and the white residue was purified by vacuum sublimation: m.p., 105° C.; reported m.p., 105° C. (6). Isopropyl P^{32}-methylphosphonic acid was synthesized from radioactive sarin* as described elsewhere (7). All other compounds were obtained commercially.

An active phosphorylase preparation was made from rat muscle according to the directions of Cori, Colowick, and Cori (4).

Whatman No. 1 filter paper sheets were used for one-dimensional ascending chromatography. The solvents used were acetic acid, *n*-butanol, and water (2); temperature, 21° C. All solutions were deionized with Amberlite cation exchange resin IR-120H in ice-cold solution before application to the filter

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**Supplied by the Chemistry Section, Suffield Experimental Station. Approximate specific activities of two batches of sarin were 300 and 800 μc./mgm.*

paper sheets. Prior to deionization, proteinous solutions were deproteinized by the addition of an equal volume of ethanol, flash evaporation of the ethanol, and centrifugation.

The presence of P^{32} on the dried sheets was detected by means of film darkening (Kodak Super XX), about 10^8 β-particles per spot providing good contrast and about 10^{10} β-particles giving heavy overexposure. Phosphate, methylphosphonate, isopropyl methylphosphonate, and their sugar esters, if any, were detected by means of the molybdate test for phosphate esters (3). The blue spots obtained with methylphosphonate and isopropyl methylphosphonate cannot be differentiated by eye from that obtained with phosphate. However, isopropyl methylphosphonate is not detectable by means of the common tests for phosphate (3). Isopropyl methylphosphonate was also detected on the untreated dried paper chromatograms by its fluorescence in the dark under ultraviolet light.

Reducing sugars were determined quantitatively in solution by the method of Somogyi (10).

Experimental and Results

P^{32}-Methylphosphonic acid (31 mgm.) was injected intraperitoneally into an adult male Wistar rat. During the first 48 hr. after injection, 92% of the administered radioactivity was excreted in 34 ml. of urine. Isopropyl P^{32}-methylphosphonic acid (56 mgm.) was injected intraperitoneally into a second rat. Here, about 40% of the radioactivity was excreted in 12 ml. of urine during the first 48 hr. after injection. Chromatographs of aliquots of these two urine samples are shown in Fig. 1, columns 1 and 3, respectively. In columns 2 and 4, aliquots of the two urine samples have been co-chromatographed with P^{32}-methylphosphonic and isopropyl P^{32}-methylphosphonic acids, respectively. The relatively large amount of solute in urine precluded the possibility of identification by ultraviolet fluorescence or molybdate staining.

The incubation of phosphate with glycogen, adenylic acid, and phosphorylase preparation according to the directions of Cori, Colowick, and Cori (4) resulted in the synthesis of a considerable amount of both glucose-1-phosphate and glucose-6-phosphate as shown chromatographically in Fig. 1, column 5. Simultaneously, using the same enzyme preparation and under the same conditions except that P^{32}-methylphosphonate and isopropyl P^{32}-methylphosphonate were used in place of phosphate, no evidence was found for the synthesis of hexose phosphonate esters. These results are shown in Fig. 1, columns 6 and 7. Even when the autoradiographic plates were heavily overexposed, no film darkening was found other than a slight enlargement of the spots shown. These experiments were repeated several times with the same results.

Column 8, Fig. 1, is a test strip for purposes of comparison and shows the chromatographic separation of (top to bottom) isopropyl methylphosphonic acid, methylphosphonic acid, phosphoric acid, glucose-6-phosphoric acid, and glucose-1-phosphoric acid.

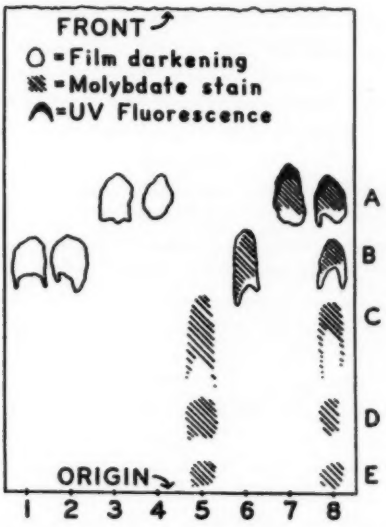

FIG. 1. The chromatographic separation of some phosphates and phosphonates. Columns 1 to 7 explained in text. Column 8 shows the separation of (*A*) isopropyl methylphosphonic acid, (*B*) methylphosphonic acid, (*C*) phosphoric acid, (*D*) glucose-6-phosphoric acid, (*E*) glucose-1-phosphoric acid.

During the incubations of glycogen, adenylic acid, and phosphorylase preparation with phosphate, with methylphosphonate, with isopropyl methylphosphonate, and in the complete absence of any phosphorus-containing anion, aliquots of the incubating solutions were removed and analyzed for reducing

TABLE I

FORMATION OF REDUCING SUGARS FROM GLYCOGEN

Anion	% Glycogen hydrolyzed to reducing sugar
Control	0
Phosphate	16
Methylphosphonate	0
Isopropyl methylphosphonate	0

sugars. The results of these analyses are shown in Table I. Analyses performed after two and four hours of incubation did not differ significantly. Also, the presence of adenylic acid at the extremely low concentration used or its omission altogether did not affect the controls wherein the hydrolysis of glycogen remained consistently at zero. The percentage of glycogen changed to reducing sugar represents the extent to which the respective anions have catalyzed, via unstable intermediates, the formation of reducing sugars from glycogen.

Discussion and Conclusions

Methylphosphonic acid does not appear to be subject to enzymatic degradation in the intact rat. Administered methylphosphonic acid is excreted in the rat's urine as such. Administered isopropyl methylphosphonic acid is also excreted in the rat's urine as such. This latter observation confirms an earlier conclusion (7).

The results shown in Table I indicate that, unlike arsenate (8), methylphosphonate and isopropyl methylphosphonate are unable, in the presence of phosphorylase enzyme, to catalyze the formation of reducing sugars from glycogen via unstable intermediate hexose esters. Fig. 1 shows that rat muscle phosphorylase is unable to catalyze the synthesis of stable hexose phosphonate esters from the corresponding phosphonic acids and glycogen. At the same time, the results of Fig. 1 and Table I show, in agreement with the mass of published information, that phosphate and rat muscle phosphorylase permit the formation of glucose-1-phosphate, glucose-6-phosphate, and reducing sugar from glycogen. Glucose-6-phosphate and reducing sugar are probably synonymous in these experiments (4).

The inability of isopropyl methylphosphonic acid to form hexose phosphonate esters is not surprising. The same resonance which stabilizes this half-acid half-ester (7) probably hinders its attack on glycogen. The apparent inability of methylphosphonic acid to form hexose esters under enzyme catalysis may be related in some way to its smaller dissociation constants: methylphosphonic acid, $pK'_{a1} = 2.38$, $pK'_{a2} = 7.74$ (5); phosphoric acid, $pK'_{a1} = 1.97$, $pK'_{a2} = 6.82$ (9). The possible biological significance of the acid strengths of phosphoric acid and some phosphoric acid esters has been discussed at some length by Kumler and Eiler (9). Knowledge obtained from the chemical synthesis of some hexose phosphonate esters (to be undertaken in the near future) may permit a profitable contribution to such a discussion.

Acknowledgments

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CARDIOVASCULAR EFFECTS OF PENTOLINIUM BITARTRATE IN DOGS[1]

By W. E. G. A. Spoerel[2] and C. W. Gowdey

Abstract

Pentolinium (5 mgm./kgm.) injected intravenously into dogs anesthetized with pentobarbital caused the pulse rate to approach that of dogs with surgical cardiac denervation. The higher the initial pulse rate, the greater the decrease after the drug; rates under 100/min. were increased. Changes in arterial pressure followed a similar pattern, and the changes in both systemic and pulmonary arterial pressures were correlated with the changes in pulse rate. The cardiac output was decreased. Pressor responses to injected adrenaline and noradrenaline were greater after pentolinium. Total peripheral resistance, respiratory rate, respiratory minute volume, and oxygen consumption were not changed significantly, but local (hind-leg) resistance was decreased in two of three experiments. Pentolinium abolished or reduced markedly the cardiovascular responses to reduced carotid sinus pressure, tilting, acute hypoxia, large doses of acetylcholine, and hemorrhage. The cardiac vagus and the cardiovascular part of the sympathetic nervous system are blocked, but the experiments suggest that the adrenal medulla may not be completely blocked by 5 mgm./kgm. pentolinium.

Introduction

A new ganglionic blocking agent, pentamethylene-1,5-bis(1-methylpyrrolidinium), the bitartrate salt of which is known as "M. & B. 2050A" or "Ansolysen", has been described by Wien and Mason (9, 21, 22). They found that it was about five times as active on the superior cervical ganglion of the cat as hexamethonium and that the block persisted longer. Clinical studies in hypertensive patients (5, 10, 11, 13, 16, 17) and in controlled hypotension during anesthesia (3) confirmed the greater potency and longer duration of action of pentolinium. Because of these potential advantages of the drug, it was decided to investigate further its cardiovascular effects; some of the results have been presented in abstract form (7).

Methods

Nineteen healthy, mongrel dogs weighing between 7.3 and 21.4 kgm. were anesthetized with sodium pentobarbital after being deprived of food for 18 hr. Anesthesia was maintained by intramuscular or intravenous pentobarbital as required. In the two-hour period from the beginning of anesthesia the dog was prepared for various cardiovascular and respiratory measurements. A Magill endotracheal tube was inserted, secured by inflation of its cuff, and connected through a short plastic tube fitted with one-way valves to a Tissot spirometer. Oxygen consumption was determined with the animal breathing room air, from measurements of the volume of expired air and the concentration of oxygen in aliquots of this expired air, measured with a Beckman oxygen

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analyzer. A radio-opaque cardiac or plastic ureteral catheter was introduced, with the aid of a fluoroscope, into the pulmonary artery through the left external jugular vein and connected to a saline manometer. This catheter allowed withdrawal of samples of mixed venous blood and also measurement of pulmonary arterial pressure (zero level—5 cm. above table top). The femoral arterial pressure and pulse rate were recorded on a kymograph with a student's mercury manometer or on a multichannel recorder with a Sanborn electromanometer. Arterial and mixed venous blood samples were withdrawn simultaneously over a one-minute period into greased heparinized syringes, and the oxygen and carbon dioxide contents were determined by the method of Van Slyke and Neill within 20 min. of withdrawal. Hematocrit levels were obtained from arterial blood samples spun at 2500 r.p.m. in Wintrobe tubes for 30 min. These measurements allowed calculation of the cardiac output (direct Fick method), stroke volume, total peripheral resistance, and pulmonary resistance.

After a suitable control period pentolinium bitartrate (5 mgm./kgm.) was injected intravenously; this injection was followed 45 min. later by a constant infusion of pentolinium bitartrate in saline at the rate of 3 mgm./kgm./hr. The cardiac output and other indices were determined before and 30, 90, and 120 min. after the initial pentolinium injection.

In 13 experiments the effects of intravenous injections of 0.5–1.0 μgm./kgm. adrenaline (Suprarenin bitartrate) and noradrenaline (Levophed bitartrate) on the heart rate, measured with the cardiochronograph, and arterial pressure were compared before and after the administration of pentolinium.

The local peripheral (hind-leg) resistance was measured in three additional dogs by a modification of the method described by Girling (6).

The blocking activity of pentolinium on various cardiovascular reflexes was measured in three dogs under the following conditions. The dog was anesthetized, fitted with an endotracheal tube, and prepared for the recording of arterial pressure and pulse rate as described above. The vagus nerves were sectioned in the neck, 0.5 mgm./kgm. atropine sulphate were injected intravenously, the animal was tied to a board so that it could later be tilted above the operating table, and loose ligatures were placed around both common carotid arteries. A battery of four tests was then performed: (1) clamping both common carotid arteries below the sinus for 45 sec., (2) tilting the dog (head up) to 85° from the horizontal for 60 sec., (3) injecting 0.3 mgm./kgm. acetylcholine intravenously, and (4) allowing the dog to breathe pure nitrogen for 75 sec. from a Douglas bag. The responses of the pulse rate and arterial pressure to these tests were measured several times before, and repeatedly after, one intravenous injection of 5 mgm./kgm. pentolinium bitartrate. In one experiment the tests were performed at least every hour for six hours following the injection of pentolinium.

The vasomotor responses to hemorrhage were studied in five control and eight pentolinium-treated dogs. Fifty milliliters of blood were withdrawn

from the femoral artery every three minutes until either (*a*) the arterial pressure had fallen below 40 mm. Hg, or (*b*) the volume of the hemorrhage approached one-third of the calculated blood volume.

The statistical significance of the various results was obtained by using Fisher's tables of "*t*" (4).

Results

The effects of pentolinium on some of the important cardiovascular–respiratory indices are summarized in Table I. The table includes only those results obtained 30 min. after pentolinium; the 90 and 120 min. readings are not significantly different from those at 30 min.

There was a significant decrease (mean—16.7 beats/min.; $P < .02$) in pulse rate 30 min. after the injection of pentolinium. Further analysis of the results showed that in 14 of the dogs the initial pulse rate varied between 123 and 198/min. and was reduced significantly ($19.0 \pm 3.37\%$) by pentolinium. The initial rate in three dogs (Nos. 2, 6, 12) was below 100/min. and in these the rate was increased by pentolinium; in dog 13 the initial rate of 112/min. was unchanged. Thus it is seen that the drug decreases a high rate and increases a low rate; the coefficient of correlation of the pulse rate before pentolinium and the rate after (as % of the rate before) was -0.87, $P < .01$.

Pentolinium produced a significant fall in arterial pressure (25.4 ± 6.03 mm. Hg) which occurred within a few minutes after the injection (Fig. 1). This fall is also shown in Table II: pentolinium decreased the mean arterial pressure from 112 ± 4.5 to 85 ± 5.5 mm. Hg ($P < .01$). It was found in other experiments that once the arterial pressure had been lowered by pentolinium it could not be further reduced by additional injections of the

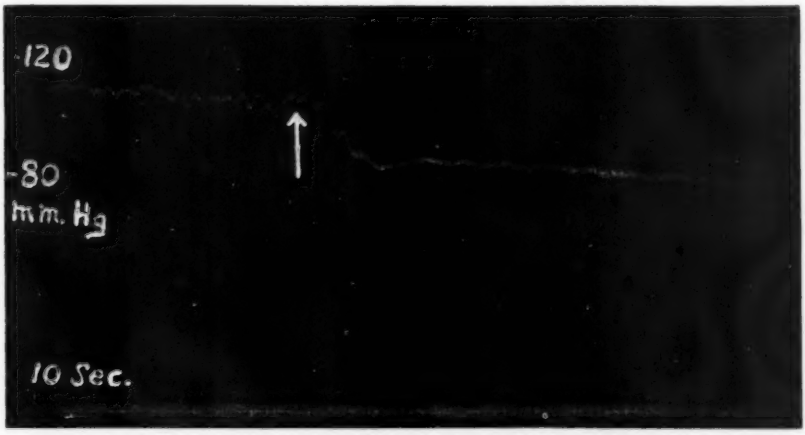


FIG. 1. Arterial pressure of dog anesthetized with sodium pentobarbital; at the arrow, 5 mgm./kgm. pentolinium bitartrate was injected intravenously. Note the diminution of the blood-pressure waves.

TABLE I

SUMMARY OF CHANGES IN CARDIOVASCULAR–RESPIRATORY INDICES AFTER PENTOLINIUM IN 19 DOGS

	Pulse rate (beats/min.)	Cardiac output (l./min.)	Stroke volume (ml.)	Arterial pressure (mm. Hg)	Total peripheral resistance (units*)	Pulmonary arterial pressure (mm. H_2O)	Pulmonary resistance (units†)	Hematocrit (%)	Oxygen consumption (cc./min.)	Oxygen utilization‡ (%)	Respiratory rate (per min.)	Respiratory minute volume (cc.)
Before pentolinium (mean and S.E.M.)	138.1 ± 6.48	1.353 ± 0.121	9.8 ± 0.88	120.1 ± 3.92	8520 ± 1270	181.5 ± 8.15	139.9 ± 8.49	46.9 ± 1.43	72.1 ± 5.10	31.3 ± 1.62	18.8 ± 2.41	2204 ± 201
30 min. after pentolinium (5 mgm./kgm.)	121.4 ± 3.06	1.155 ± 0.097	9.2 ± 0.83	94.7 ± 4.58	7548 ± 888	176.2 ± 8.42	173.3 ± 19.18	45.6 ± 1.22	68.8 ± 1.34	36.0 ± 1.35	23.1 ± 2.86	2821 ± 284
P value of difference	< .02			< .01								

*$TPR\ units\ calculated\ as \dfrac{mean\ art.\ press.\ (mm.\ Hg) \times 1332}{cardiac\ output\ (ml./sec.)}$.

†$Pulm.\ resis.\ units\ calculated\ as \dfrac{mean\ pulm.\ art.\ press.\ (mm.\ H_2O)}{cardiac\ output\ (l./min.)}$.

‡$Oxygen\ utilization\ is \dfrac{arteriovenous\ oxygen\ difference}{arterial\ oxygen\ content} \times 100$.

drug. In addition to the fall in arterial pressure, Fig. 1 also illustrates another common observation: the disappearance of the blood-pressure waves usually considered (23) to be the result of changes in vasomotor tone. The respiratory waves were not abolished. The two dogs which showed an increased arterial pressure after pentolinium had low initial pulse rates (< 100 per min.).

There was a significant correlation ($r = 0.79$, $P < .01$) between the pulse rate and arterial pressure after pentolinium (both calculated as % of control levels) and between the pulse rate and pulmonary arterial pressure ($r = 0.83$, $P < .01$). There was also a significant correlation between the arterial pressure before pentolinium and the fall in blood pressure produced by the drug ($r = 0.53$, $P < .05$); in other words, the higher the initial blood-pressure level the greater was the fall.

TABLE II

POTENTIATING EFFECT OF PENTOLINIUM ON THE PRESSOR RESPONSES TO ADRENALINE AND NORADRENALINE

	Noradrenaline			Adrenaline		
	Before Pentolinium	After Pentolinium	P value	Before Pentolinium	After Pentolinium	P value
No. of dogs	13	13	—	5	5	—
B.P. (mm. Hg) before catechol amine injection	112 ± 4.5*	85 ± 5.5	<.01	113 ± 2.7	91 ± 6.3	<.02
Highest B.P. recorded	146 ± 2.2	151 ± 2.6	—	137 ± 7.7	149 ± 11.8	—
Mean rise in B.P. (mm. Hg)	34 ± 3.0	66 ± 4.4	<.001	24 ± 5.4	58 ± 7.0	<.01
Duration of pressor response (sec.)	111 ± 16.8	178 ± 14.8	<.01	78 ± 13.7	168 ± 7.9	<.01

**Mean and standard error of mean.*

The mean decrease in cardiac output 30 min. after pentolinium was 0.197 ±.089 liters/min.; this was significant at the 5% level.

Pentolinium did not change significantly the stroke volume, oxygen consumption, respiratory rate and volume, hematocrit level, pulmonary resistance, and total peripheral resistance. The resistance in the hind leg was, however, reduced in two of three experiments.

The consistent increases in the magnitude and duration of the pressor effects of adrenaline and noradrenaline after pentolinium are illustrated in Fig. 2 and Table II. It is evident that the typical bradycardia produced by noradrenaline is replaced by a marked tachycardia after pentolinium; the maximum arterial pressures induced by the pressor agents, however, are not different from those in the control period.

Table III shows that with a significantly smaller loss of blood the arterial pressure of the treated animals fell significantly more than that of the controls.

Figs. 3, 4, 5, and 6 illustrate the blocking effect of pentolinium on various pressor reflexes. In every experiment the blockade is known to have persisted for more than three hours; in one it lasted more than six hours.

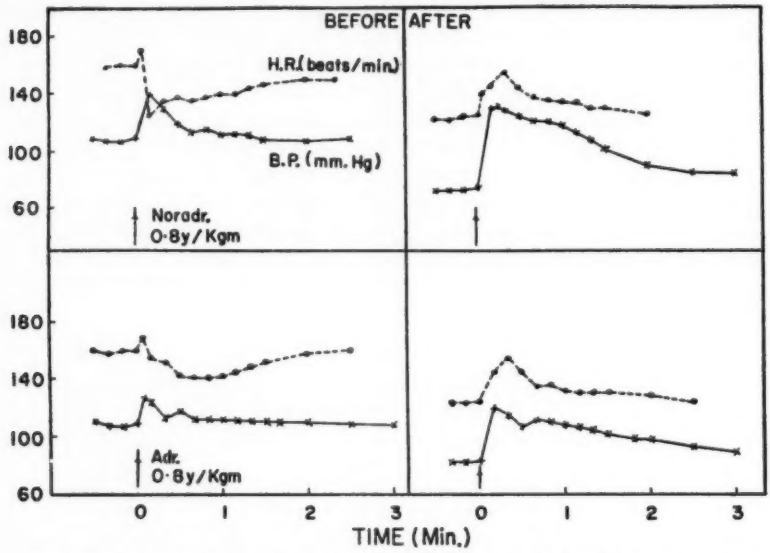


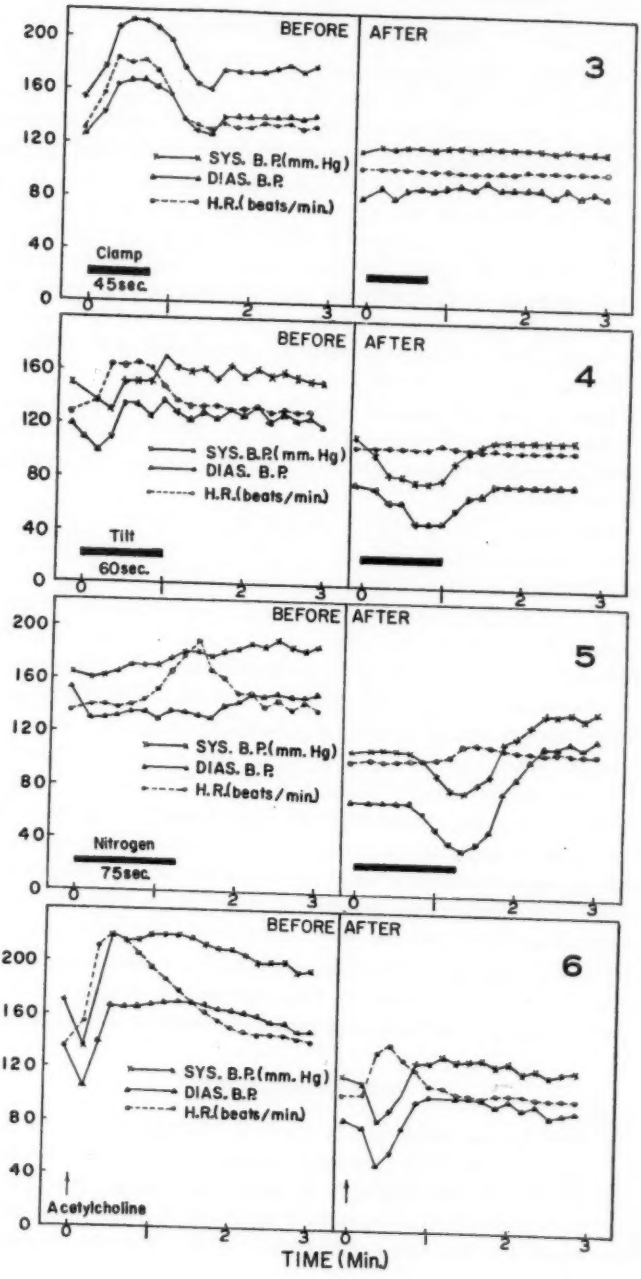
FIG. 2. Changes in heart rate and mean arterial pressure induced in a dog by intravenous injections (at the arrows) of noradrenaline and adrenaline before and 30 min. after the intravenous administration of pentolinium bitartrate (5 mgm./kgm.). Note that the bradycardia induced by noradrenaline before pentolinium was replaced after the drug by marked tachycardia.

TABLE III

INCREASED SENSITIVITY TO HEMORRHAGE OF PENTOLINIUM-TREATED DOGS

Controls			Pentolinium (5 mgm./kgm.)		
Expt.	Volume of blood removed (ml./kgm.)	% Fall in arterial pressure	Expt.	Volume of blood removed (ml./kgm.)	% Fall in arterial pressure
1	32	58	2	21	67
3	36	35	4	18	58
5	36	24	6	9	50
7	28	19	8	22	65
9	26	2	10	13	56
			12	17	34
			14	17	45
			16	14	61
Mean and S.E.M.	31.6 ± 2.02	27.6 ± 9.39		16.4 ± 1.51	54.5 ± 3.87
P value of difference	< .01	< .02			

FIGS. 3, 4, 5, 6. Dog anesthetized with sodium pentobarbital; cervical vagi sectioned; 0.5 mgm./kgm. atropine sulphate injected intravenously. Changes in heart rate and systolic and diastolic arterial pressures induced: by clamping both common carotid arteries for 45 sec. (FIG. 3); tilting the dog (head up) to 85° from the horizontal for 60 sec. (FIG. 4); allowing the dog to breathe 100% nitrogen for 75 sec. (FIG. 5); and injecting 0.3 mgm./kgm. acetylcholine intravenously (FIG. 6) before and 30 min. after the intravenous injection of 5 mgm./kgm. pentolinium bitartrate.


BEFORE
AFTER
3
SYS. B.P.(mm. Hg)
DIAS. B.P.
H.R.(beats/min.)
Clamp
45sec.
4
Tilt
60sec.
5
Nitrogen
75sec.
6
Acetylcholine
TIME (Min.)

Discussion and Conclusions

The results show that pentolinium blocks both the cardioaccelerator and cardio-inhibitory impulses. On the one hand, a slow heart rate (<100) was increased by the drug, and the noradrenaline-induced reflex bradycardia was replaced by marked tachycardia. On the other hand, the elevated heart rate usually accompanying pentobarbital anesthesia was reduced by pentolinium. Under these conditions the heart rate is set at a new level of 121.4 ± 3.06. In other experiments in this laboratory we have found an average heart rate of 120/min. in dogs in which the vagi were blocked by procaine and the sympathetic nerves by extradural injections of procaine. In dogs with chronic complete surgical denervation of the heart, the average heart rate measured after the same duration of pentobarbital anesthesia was 105/min. The reason for this discrepancy is not known.

Reductions in arterial pressure and cardiac output were found earlier by us with blocking doses of hexamethonium (12, 19). The increased pressor response to injected adrenaline observed after pentolinium has also been shown after hexamethonium (15).

The results show clearly that pentolinium interferes markedly with the circulatory adjustments to reduced carotid-sinus pressure, acute hypoxia, pooling of blood in the extremities, large doses of acetylcholine, and hemorrhage. Tetraethylammonium chloride was shown by Brown, Wood, and Lambert (1) to block the compensatory reflex mechanism induced in human subjects by tilting and by centrifugal force. In the present experiments, the block produced by pentolinium persisted longer than that of either tetraethylammonium (1) or hexamethonium (19).

The pressor responses to acute hypoxia and to acetylcholine, although delayed and markedly reduced (Figs. 5, 6), were not completely abolished by pentolinium. Similarly, hexamethonium was shown (12) merely to delay and reduce the hypoxia-induced rise of arterial pressure. There is good evidence that both of these stimuli act finally through the adrenal medulla (2, 8, 14, 18, 20), and it is possible that the dose of pentolinium used, though sufficient to block the sympathetic ganglia, did not completely block the adrenal medulla.

The increased sensitivity of the arterial pressure of the pentolinium-treated animals to hemorrhage is similar to that reported by Wiggers (24) in dogs in which the sympathetic nervous system was blocked by dibenamine.

Acknowledgments

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THE EXTRACTABLE HEPARIN IN DIFFERENT ANIMAL TISSUE[1]

By Frank C. Monkhouse

Abstract

Heparin is extracted from tissue by 0.5 *M* potassium thiocyanate. The protein is removed from the extract with 80% phenol and the heparin precipitated from the aqueous layer with three volumes of 95% ethanol. Further purification is achieved by reprecipitating the heparin with alcohol, dissolving the precipitate in saline, and precipitating it with 5% benzidine hydrochloride. The amount of heparin extractable by this method has been determined in a variety of tissues from rabbit, dog, beef, monkey, and man. The amounts expressed in units per 100 gm. of tissue varied from less than four units in the rabbit intestine to 4800 units in the liver of a hypophysectomized dog. Radioactive heparin was obtained from dog liver 48 hr. after the animal had been fed S^{35} contained in hydrolyzed yeast. The significance of these findings in relation to further studies on the physiological role of heparin is considered.

Though recently Freeman *et al.* (3) and Nilsson (12) claim to have shown that normal plasma contains considerable amounts of heparin, Monkhouse and Jaques (11) were unable to extract any from normal blood. On the other hand heparin is present in large amounts in dog liver, beef lung, and certain tumors, and in lesser amounts in many other tissues. This suggests that heparin may have physiological functions which are not concerned with blood coagulation.

The early work of Hahn (5) and Weld (18) and the more recent work of Grossman *et al.* (4) and Shore (17) on the lipemia clearing action of heparin encourages speculations that it is involved in normal lipid transport. Riley and West (13, 15), showed that mast cells are rich in histamine as well as heparin and that both substances are released when these cells are disrupted by the action of histamine liberators. These findings have led them to suggest (14) that heparin may be concerned with events in connective tissue rather than with the coagulability of blood.

One of the difficulties in solving problems relevant to the physiology of heparin is the lack of methods for extracting it from small amounts of tissue. Since there is evidence that heparin is released from some tissue more easily than others (14) alternative methods will be valuable in helping to determine if a low value is merely the result of failure to extract the heparin.

Jaques and his colleagues (6) extract the tissue successively with saline at 100° C., then acetone and water at room temperature, and analyze the extract chromatographically. According to these authors the method can be applied to as little as 0.5 gm. of tissue. The following is a report on an alternative method for use with tissue samples of from 2–100 gm. Various modifications have been tried during the course of investigations throughout the past three years. The procedure to be described has given consistent results and is now used routinely for the extraction of heparin from all types of tissue.

[1]*Manuscript received March 21, 1956.*
Contribution from the Department of Physiology, University of Toronto, Toronto, Ontario. The work was supported by Grant No. 9310-32 from the Defence Research Board.

Methods

Heparin Extraction

The tissue is macerated in 5 ml. of 0.5 *M* potassium thiocyanate per gram of tissue, and the mixture agitated at room temperature for three hours. The extract is filtered through gauze and treated with one-half volume of 80% phenol, and allowed to stand overnight. After centrifugation, the upper aqueous layer is removed and the heparin is precipitated from it by adding three volumes of ethanol and allowing the mixture to stand for two hours at 4° C. The centrifuged precipitate is successively washed with ethanol and ether and dried. The heparin is extracted from the precipitate with saline and assayed. Extracts from beef lung and dog liver assay between 15 and 20 units per mgm. at this stage. The concentration can be increased to approximately 70 units per mgm. by reprecipitating the heparin with alcohol, extracting it with a reduced volume of saline, and again precipitating it with one-half volume of a 5% benzidine hydrochloride according to the procedure of Astrup (2).

Crystallization of heparin from 100 gm. quantities of dog liver and beef lung can be carried out by dissolving the benzidine precipitate in a suitable volume of weak ammonia water, adding barium acetate and glacial acetic acid at 65° C., and slowly cooling the mixture.

Heparin Assay

Heparin extracts, unless otherwise stated, were assayed according to their ability to prevent the coagulation of citrated dog's blood on the addition of dilute solutions of thrombin as first described by Jaques and Charles (7). Since it is known that heparin from different species may show marked differences in specific activity (8), heparin activity throughout this paper is expressed in terms of standard beef heparin kindly supplied by the Connaught Medical Research Laboratories. The metachromatic assay referred to in Table I was carried out as described by Jaques, Monkhouse, and Stewart (9) using the Lovibond tintometer.

Results

Results from a typical experiment on 100 gm. of beef lung are illustrated in Table I. The first alcohol precipitate was extracted with two 4 ml. quantities of saline at pH 7.5, the extracts pooled, assayed, and the heparin again precipitated by addition of three volumes of alcohol. The precipitate was washed with alcohol, then ether, dried in a vacuum desiccator for 48 hr., and weighed. It was extracted with 2 ml. of saline at pH 7.5. The extract was assayed and mixed with 1 ml. of 5% benzidine hydrochloride. The resulting precipitate was dissolved in 1 ml. of weak ammonium hydroxide and extracted with ether. The ether was pipetted off and the heparin precipitated with four volumes of alcohol. The precipitate was dried and weighed as previously described. This time it was dissolved in 0.5 ml. of very weak ammonium hydroxide solution and 0.02 ml. was used for assay. The remainder was

TABLE I

THE CONCENTRATION OF HEPARIN IN AN EXTRACT FROM 100 GM. BEEF LUNG AT DIFFERENT STAGES OF PURIFICATION

Stage of purification	Wt. of ppt., mgm.	Units of heparin			
		Antithrombin		Metachromatic	
		Total	Units/mgm.	Total	Units/mgm.
First alcohol precipitate	—	2080	—	1920	—
Second alcohol precipitate	43	1500	35	1540	36
Benzidine hydrochloride	11	850	77	900	82

heated to 70° C. and 0.15 ml. of barium acetate added. This caused some cloudiness, which cleared when 0.10 ml. of glacial acetic acid was slowly added. The tube was allowed to cool in the water bath to room temperature and was then kept at 4° C. for a further two hours. On examination of the precipitate, typical heparin crystals were observed. This type of experiment has been repeated with 100 gm. samples of beef lung and dog liver.

It can be seen from the data presented that over 50% of the heparin is lost during the purification process. While the example presented shows a slightly greater than average loss, it has so far been impossible to obtain more than a 70% recovery at the end of the benzidine precipitation stage. For this reason, especially when the tissue used has a low heparin level such as is found in the rabbit, rat, guinea pig, monkey, and man, the value obtained by antithrombin assay after the first alcohol precipitate is taken for extractable heparin. The extract at this stage is crude but assays on duplicate samples are generally in good agreement.

TABLE II

EXTRACTABLE HEPARIN LEVEL IN VARIOUS ANIMAL TISSUES

Species	Organ	Units/100 gm. tissue	No. of animals
Rabbit	Liver	16–28	6
	Lung	12–16	6
	Kidney	36–54	6
	Intestine	1–4	6
Dog	Liver	760–1800	10
	Lung	440–500	3
	Kidney	200–520	6
	Heart	160–510	9
Man	Liver	28	1
	Tumor*	250	1
Monkey	Liver	95	1
Ox	Lung	1600–2100	5

NOTE: *Each determination was carried out in duplicate, 5 gm. of tissue being used for each determination.*

**Ovarian tumor rich in mast cells.*

In Table II, the extractable heparin content of different animal tissues i shown. No values are presented for tissues of the rat or guinea pig, but experiments on tissues from these animals showed their heparin content to be low. Thus of the laboratory animals usually available, the dog is the only one with a high heparin level. The liver of this species may contain 100 times as much heparin as an equivalent weight of rabbit, rat, or guinea pig liver. The only tissue other than an ox lung which approached dog tissue in heparin content was an ovarian tumor from a human patient. This tumor was rich in mast cells.

In Table III results are given for the extraction of the liver of a dog which had been fed 0.61 mc. of S^{35} contained in 11 mgm. of hydrolyzed yeast 48 hr. before the animal was sacrificed. The ratio of radioactivity per unit of heparin remained constant during a fourfold purification of the extract. This material was injected intravenously into a rabbit and the blood heparin levels measured at intervals. The rate of decrease in heparin level was similar to the decrease when a corresponding amount of commercial beef heparin was injected, and the biological activity and radioactivity decreased at an equal rate. Thus there is good evidence that the S^{35} was incorporated into the heparin molecule. Radioactive heparin was also obtained from a dog which was fed 0.79 mc. of S^{35} contained in 40 mgm. of *l*-methionine.

TABLE III

HEPARIN FROM THE LIVER OF A DOG WHICH HAD RECEIVED 0.61 MC. OF S^{35} 48 HR. EARLIER

Stage of purification	Wt. of ppt., mgm.	Counts/min.		Heparin units		Counts/ min./unit
		Total	Per mgm.	Total	Per mgm.	
First alcohol precipitate	87.2	5978	68.6	1500	17	4.0
Benzidine hydrochloride	13.9	4350	313.0	1000	72	4.4

During the course of these investigations an opportunity arose to study the heparin levels in normal and hypophysectomized dogs, some of which had received growth hormone. We are indebted to Dr. James Campbell of this department for making available these tissue samples. Growth hormone was given twice daily in the dose of 1 mgm./kgm., body weight/day. Control animals were given saline in place of growth hormone under the same conditions. The tissue was frozen and kept at $-20°$ until extracted. In Table IV, the results are given for five normal and seven hypophysectomized animals. In spite of the relatively large variation between animals of the same experimental group there is a noticeably higher heparin level in the hypophysectomized animals. Growth hormone does not appear to affect the level in either the normal or hypophysectomized animals. The difference in level of heparin between normal and hypophysectomized dogs is highly significant at the 1% level.

TABLE IV

EXTRACTABLE HEPARIN LEVEL IN THE LIVERS OF HYPOPHYSECTOMIZED DOGS

Treatment	Heparin units/100 gm. tissue	
	First alcohol ppt.	Benzidine HCl ppt.
N + G.H.	1200	800
N + G.H.	720	640
N	1800	—
N	1660	—
N	760	—
H	3680	—
H	2800	1400
H + G.H.	1600	1440
H + G.H.	4800	3040
H + G.H.	2720	2280
H + G.H.	3100	—
H + G.H.	2000	2000

NOTE: *N—Normal dog. G.H.—Given growth hormone. H—Hypophysectomized.*

Discussion

The low heparin level found in the tissues of many animals is one of the difficulties in the study of the physiology of heparin. Of the laboratory animals examined in this series, the dog is the only one in which significant amounts of heparin can be obtained from small quantities of tissue. The question of whether the low level is a result of the inability to extract the heparin rather than a true lack of the substance cannot be adequately answered at this time. By the method used in these experiments it is possible to extract heparin from dog liver and beef lung in quantities comparable to those extracted by previously published methods and, when samples as large as 100 gm. are used, the crystalline barium salt of heparin can be obtained. Furthermore the ratio of the heparin levels in the blood of rabbits and dogs (10) undergoing anaphylactic shock compares well with the ratio of the heparin content of their tissues as determined by the method here described.

The increase in the amount of extractable heparin in the liver of hypophysectomized dogs as compared to controls suggests that some metabolic pathway involving heparin is influenced by the pituitary gland. The work of Asboe-Hansen (1), who found that the number of mast cells in dermal connective tissue of man, rabbit, and guinea pig decreased after the administration of cortisone, or ACTH, suggests that the pituitary may act through the adrenal cortex. Schock and Glick (16) on the other hand were unable to show any significant change in the mast cells of rat skin on the administration of ACTH or cortisone. The findings that radioactive heparin can be obtained by feeding S^{35} to dogs may open up another avenue of approach to this problem.

Summary

A method for the extraction of heparin from small quantities of tissue has been developed. Using this method the amount of extractable heparin in different tissues of rabbit, dog, monkey, and man was determined. On the basis of the antithrombin assay 100 times more heparin could be extracted, per gram of tissue, from dog liver than from the livers of rat, rabbit, guinea pig, monkey, or man. Radioactive heparin was obtained from the livers of dogs fed S^{35} contained in yeast hydrolyzate or *l*-methionine. The heparin content of the livers of hypophysectomized dogs was found to be significantly higher than that of normal controls.

Acknowledgments

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THE EFFECT OF INSULIN ADMINISTRATION ON ARTERIOVENOUS GLUCOSE DIFFERENCES IN THE ALLOXAN-DIABETIC DOG[1]

By Doris E. Gray[2] and H. A. DeLuca[3]

Abstract

It has been reported by others and confirmed by the present workers that following the intravenous administration of insulin in the normal animal, the glucose concentration in venous blood may exceed that of arterial blood giving rise to a negative arteriovenous difference. The simultaneous administration of glucose enhances this effect. The present investigators have extended this work to the alloxan-diabetic animal (dog) and have shown that large negative arteriovenous blood glucose differences are obtained following administration of insulin and glucose. These differences are too great and too consistent to allow the contention that negative arteriovenous differences are simply the result of analytical errors.

Introduction

Henriques and Ege (6) and Foster (3) were among the earliest investigators to undertake a study of the arteriovenous difference (A-V difference) in blood glucose concentration as an index of the utilization of this carbohydrate by the peripheral tissues. Since that time, numerous workers have extended these studies under a variety of conditions. There are reports, however, of instances in which the concentration of glucose in venous blood exceeded that of the arterial blood, resulting in a negative A-V difference. For example, Norgaard and Thaysen (11), Friedenson *et al.* (4), and Bell and Burns (1) reported instances in which the A-V difference became negative following insulin administration. Other investigators, such as Griffiths (5), Lawrence (7), and Mosenthal (9), found negative A-V differences in fasting diabetic subjects. On the other hand, Somogyi (13) has expressed the opinion that negative A-V differences are not real entities but undoubtedly arise as the result of analytical errors.

In some preliminary work using the normal dog as the experimental animal, the results obtained by the present authors appeared to confirm the occurrence of negative A-V differences following administration of insulin. Under conditions similar to those outlined below, five out of six animals showed negative A-V differences ranging from −2 to −7 mgm. per 100 ml. blood (mean −4 mgm.%). Admittedly these differences are not great. There were no negative values in the fasting A-V differences, which varied from 0 to 10 mgm.% (mean 5 mgm.%). Simultaneous administration of insulin and glucose in four animals produced larger negative A-V differences, ranging from −6 to −14 mgm.% (mean −10 mgm.%). Again there were no negative values when the animals were in the fasting state. The values ranged from 3 to 15 mgm.% (mean 7 mgm.%).

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Friedenson *et al.* (4) have pointed out that a negative A-V difference obviously implies that glucose is given off by the tissues to the blood. Presumably after insulin administration, the level of glucose in the blood stream falls temporarily below that of the interstitial fluid. A back diffusion of glucose from the latter into the blood stream takes place, thus raising the sugar level at the venous end of the capillary compared to the arterial end. This gives rise to a negative A-V difference. In view of this explanation, it was considered of interest to investigate the effect of insulin and glucose on the A-V difference in the alloxan-diabetic animal, in which the glucose level is raised in both the blood and interstitial fluid. If such an explanation were correct, the administration of insulin and glucose should cause a greater negative A-V difference than that observed in normal animals.

Method

In order to facilitate the obtaining of repeated arterial blood samples, the carotid artery of the animal was brought to the outside in the form of the so-called "carotid-loop". The animal was made diabetic with alloxan. In subsequent experiments, the arterial and venous (short saphenous vein) samples were taken under nembutal anesthesia, since the preliminary experiments showed that it was difficult to obtain simultaneous samples in the unanesthetized dog.

Arterial and venous samples were taken in the fasting state for glucose estimation. The animal was then given intravenously a 5% glucose solution on the basis of 1 gm. of glucose per kgm. body weight. Arterial and venous samples were taken for glucose estimation at various intervals over a three-hour period. Similar determinations were carried out following:

(1) The intravenous injection of crystalline zinc insulin on the basis of 0.5 units per kgm. body weight.

(2) The intravenous injection of glucose and insulin on the basis previously described. The insulin injection followed immediately after the glucose administration.

The Nelson (10) modification of the Somogyi method (12) was employed for glucose determinations. The values reported here are the means of duplicate determinations.

Results

In Table I is shown a typical set of blood sugar values for the six diabetic animals in the fasting state. In Table II are shown the lowest values for the A-V differences (the greatest values for negative A-V differences) obtained in the diabetic animals under the experimental conditions indicated. Five of the six animals receiving insulin only showed negative A-V differences, which were most marked at approximately 120 min. after insulin injection. The fasting A-V differences of these animals just prior to insulin administration are also included. A statistical analysis of the lowest A-V differences following insulin injection and the fasting levels shows that insulin administration tends to produce or increase negative A-V differences ($P < 0.02$). The same conclusion

TABLE I

FASTING BLOOD GLUCOSE LEVELS IN ALLOXAN-DIABETIC ANIMALS

Animal	Arterial, mgm. %	Venous, mgm. %	A-V difference, mgm. %
1	127	128	−1
2	366	362	4
3	161	150	11
4	124	114	10
5	760	766	−6
6	99	101	−2

NOTE: *Results expressed as mgm. of glucose per 100 ml. of blood.*

TABLE II

A-V DIFFERENCES IN ALLOXAN-DIABETIC DOGS

Animal	Fasting	Insulin	Difference	Glucose	Glucose and insulin	Difference
1	−1	−6	5	0	−12	12
2	4	−5	9	6	−71	77
3	11	−3	14	9	−13	22
4	10	1	9	0	−30	30
5	−6	−28	22	8	−34	42
6	−2	−5	3	−9	−14	5
			Mean ± S.E.M. = 10.3±2.9			Mean ± S.E.M. = 31.3±10.6

NOTE: *Insulin given on basis of 0.5 units per kgm. body weight. Glucose administered as 5% solution on basis of 1 gm. glucose per kgm. body weight. Results expressed as mgm. of glucose per 100 ml. blood.*

is reached by an analysis of A-V differences following glucose administration and that following glucose and insulin administration ($P < 0.05$). The effect of insulin in the latter instance was most marked at approximately 65 min. The mean A-V difference for different times after injection for the diabetic animals receiving (i) glucose (ii) glucose and insulin is shown in Fig. 1. The capacity of insulin to produce negative A-V differences is clearly indicated in this figure.

Discussion

The data presented above suggest that the administration of insulin with or without glucose tends to produce negative ateriovenous blood glucose differences. As already pointed out, these differences in the normal animal are small for the most part, but in the case of the diabetic animal after the simultaneous administration of insulin and glucose, they are quite large. These data would suggest that negative arteriovenous glucose differences do occur under certain experimental conditions and that they are not necessarily the result of analytical errors as suggested by Somogyi (13). In his experiments, Somogyi (13) used human subjects weighing for the most part 60 to 75 kgm. In two sets of experiments he gave intravenous doses of insulin of

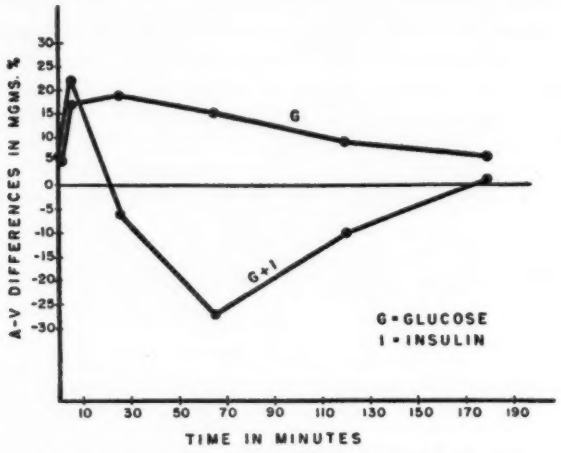

FIG. 1. A-V glucose differences in alloxan-diabetic dogs.

3 and 6 units respectively. Expressed on a weight basis, these dosages represent approximately 0.05 and 0.1 units per kgm. body weight. In the present investigation, the animals were given an insulin dosage of 0.5 units per kgm. body weight. Notwithstanding Somogyi's objection to expressing insulin dosage on a weight basis (13), it is obvious that the animals in the present investigation received relatively much larger doses of insulin. Interestingly enough, the data from Somogyi's experiments (13) show that he obtained smaller positive A-V differences in some instances with the 6 units of insulin than with the 3 units. One is accordingly tempted to speculate that with increasing dosages of insulin, the A-V differences might have continued to shrink, possibly even becoming negative. Norgaard and Thaysen (11), using 12 units of insulin in human subjects, report negative A-V differences, but the latter are not large and there is no indication that they are statistically significant. On the other hand, Bell and Burns (1) obtained negative A-V differences in human subjects using only 0.013 units of insulin per kgm. body weight. Their negative A-V differences are quite small, although the authors indicate that they are statistically significant.

In a subsequent series of experiments (14), in which insulin and glucose were administered to the same subject, Somogyi fails to report any negative A-V differences. Here again small doses of insulin were employed. The glucose was administered orally. It must be pointed out that the various reports of Somogyi (13, 14) were carried out on normal (human) subjects. Although the present authors did obtain negative A-V differences in the normal dog, the greatest differences were found in the diabetic animal.

The existence of a negative A-V difference assumes that under certain conditions, glucose can pass from the interstitial fluid into the capillaries, thus giving rise to a higher value of blood sugar in the vein than that in the corresponding artery (4). The mechanism that produces this state of affairs

is possibly as follows. Intracellular carbohydrate in the peripheral tissues is not immediately available as glucose. However, glucose in the interstitial space conceivably could diffuse back into the blood stream, if its concentration in the latter fell below that of the interstitial fluids. Insulin causes a lowering of blood sugar in both the liver and the peripheral tissues by promoting the deposition of glycogen. The passage of glucose into peripheral tissues was demonstrated by the experiments of Mann and Magath (8), who showed that insulin causes a lowering of the blood sugar even after total removal of the liver. In such animals, the lowering of the blood sugar is not nearly as pronounced as in the intact animal. In other words, although both the liver and the peripheral tissues tend to lower blood sugar under the action of insulin, the liver effect is more marked. Furthermore it has been shown by Bouckaert and De Duve (2) that the higher the dose of insulin above physiologically-secreted amounts, the more it tends to act on the liver rather than on the peripheral tissues. (In some of their experiments they obtained a ratio of 25 : 1 for hepatic action versus peripheral action.) These conclusions suggest that the administration of insulin causes a greater lowering of blood glucose on the arterial side than occurs in the interstitial fluid surrounding the peripheral tissues. Since now the glucose concentration in the latter exceeds that of arterial blood, a back diffusion of glucose into the blood stream occurs in order to re-establish the steady state. Furthermore, the intravenous administration of glucose temporarily raises the concentration in the extracellular fluid, a factor which likewise tends to increase negative A-V differences when insulin is administered.

In the case of the diabetic animal, not only is the glucose concentration of the extracellular fluid higher than normal, but the lowering of glucose on the arterial side, as a result of insulin administration, is likewise more pronounced. These two factors both act in the direction of greater negative A-V differences.

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STUDIES OF LIGNIN BIOSYNTHESIS USING ISOTOPIC CARBON

V. COMPARATIVE STUDIES ON DIFFERENT PLANT SPECIES[1]

By Stewart A. Brown[2] and A. C. Neish

Abstract

Seven species of higher plants have been tested for their ability to convert radioactive tyrosine to lignin *in vivo*. A total of 11 species representing 10 families have now been compared and only *Triticum vulgare* and *Calamagrostis inexpansa*, both members of the Gramineae, have given positive results. It is suggested that lack of a specific enzyme system may prevent lignin formation from tyrosine in the other species. The metabolic differences appear to be restricted to tyrosine utilization, for both wheat and maple have similar abilities to use a number of labelled cinnamic acid derivatives, as well as phenylalanine. Further evidence is presented that each type of lignin polymer has a corresponding aromatic monomer. Sinapic acid, ferulic acid, and probably *p*-hydroxycinnamic acid are preferentially transformed to syringyl, guaiacyl, and *p*-hydroxyphenyl lignins respectively.

Introduction

Earlier papers of this series (1–3, 17) have described tracer investigations of lignin formation by higher plants *in vivo*. The simplest labelled organic compound which was transformed to lignin was shikimic acid (1). By analogy to the reactions found by Davis and co-workers in bacteria (8), the participation in aromatic biosynthesis of a number of compounds biologically related to shikimic acid has been inferred for higher plants.

Among the aromatic compounds studied, those with a phenylpropane (C_6, C_3) skeleton were in general readily converted to at least one type of lignin polymer. The fact that fully aromatic compounds with no substituents in the ring, such as phenylalanine and cinnamic acid, were efficiently transformed to lignins shows that formation of the aromatic ring precedes its substitution (1, 2). Furthermore, the demonstration that ferulic acid (4-hydroxy-3-methoxycinnamic acid) underwent preferential conversion to the lignin polymer which yields vanillin on oxidation strongly suggested that each type of lignin has an analogous aromatic monomer.

A marked species difference has been observed in the utilization of tyrosine. Wheat can convert phenylalanine and tyrosine to lignin equally well, but three other species of plant investigated could scarcely use tyrosine for lignin formation.

In the present study the question of species differences and similarities has been investigated further. More species have been tested for ability to use tyrosine for lignin synthesis, and two species, wheat (*Triticum vulgare* Vill.) and maple (*Acer Negundo* L.), have been compared in their ability to form lignins from a variety of aromatic monomers.

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[2]*On leave to the University Chemical Laboratory, Cambridge, England, 1955–56.*

Experimental

Cultivation of the Plants

The buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) and some of the wheat (var. Thatcher) were grown in gravel culture in the greenhouse under conditions previously described (2). The rest of the wheat was grown in a field plot. The other plants were found growing wild near Saskatoon and were used at or near the flowering stage when feasible.

Synthesis of C^{14}-labelled Compounds

Randomly labelled L-*phenylalanine* and L-*tyrosine* were purchased from Atomic Energy of Canada, Ltd. The syntheses of the β-labelled *cinnamic* and *ferulic acids* have been described (2).

The ring-substituted cinnamic acids listed in Table II were all synthesized from the corresponding derivatives of benzoic acid by the same general route, i.e. reduction to the aldehyde by Rosenmund's method (14) and condensation of the aldehyde with malonic acid (18). Carboxyl-labelled *m*-anisic acid was prepared (82% yield) by the Grignard reaction as described for *p*-anisic acid (15). Carboxyl-labelled *p*-anisic acid (1 gm.) was demethylated by refluxing with 12 ml. of acetic acid and 4 ml. of 45% hydriodic acid for five hours to give *p*-hydroxybenzoic acid in 93% yield. The benzyl ether of carboxyl-labelled vanillic acid (13) treated in the same manner gave protocatechuic acid in 84% yield. The preparation of carboxyl-labelled syringic acid has been described previously (2).

The conversion of syringic acid to acetylsinapic acid has been described by Freudenberg and Hubner (10). Their methods were followed except for the acetylation, which was carried out by the method of Chattaway (7) allowing four minutes for the reaction (yield 80%). The acid chloride was purified by one recrystallization. The reduction was carried out in a glass tube with a cold finger condenser arranged so there was no possibility of the silicone grease, used on the ground glass joints, coming in contact with the refluxing xylene. Early experiments in which this precaution was not observed gave very poor yields. Agitation was effected by a Teflon-coated magnetic stirrer. The acetylsinapic acid was deacetylated in aqueous sodium hydroxide (1 *M*) in a nitrogen atmosphere to give β-labelled *sinapic acid* (yield 93%). The over-all yield based on syringic acid was about 18%. The β-labelled *caffeic acid* and *p-hydroxycinnamic acid* were prepared from protocatechuic and *p*-hydroxybenzoic acids by the same methods. The O-acetyl acid chlorides were purified by distillation under reduced pressure (23 mm.). The acetylated aldehydes obtained from the reduction were deacetylated as described above and the hydroxyaldehydes isolated by bisulphite extraction and crystallized. The condensation with malonic acid was carried out as described previously (18). The over-all yield of each, based on the hydroxybenzoic acid, was about 18%. The β-labelled *m-methoxycinnamic acid* was obtained from the carboxyl-labelled *m*-anisic acid in an over-all yield of 26%. The acid chloride and the

aldehyde were purified by distillation under reduced pressure (54 mm.) and the condensation with malonic acid was effected using the conditions of Chakravarti and co-workers (6).

Uniformly labelled benzaldehyde, purchased from Atomic Energy of Canada Ltd., was condensed with malonic acid to give cinnamic acid labelled in the ring and β carbons. This was hydrogenated (12) at room temperature in 95% ethanol using platinum oxide catalyst to give *β-phenylpropionic acid* (yield 75%).

Administration of Labelled Compounds

The twigs or stems of the plants were cut under water with a razor blade, and the cut ends were immersed in 1 ml. of solution containing the radioactive compound. One or two cuttings (depending on size) were used in each experiment. The solution (1 ml.) was absorbed within one to two hours, although in a few cases more time was required. Then 0.5 ml. of distilled water was added. After this was absorbed the cuttings were placed, with the ends in tap water, by a north window for approximately 24 hr. L-Phenylalanine was fed to each lot of plants as a standard precursor for comparison.

Isolation of Phenolic Aldehydes

The buckwheat plants were disintegrated in a Waring blendor with hot 80% ethanol, and the residue was separated by filtration and washed with hot ethanol followed by ether. The other plants were dried at 60° *in vacuo* and extracted before oxidation as previously reported (2, 3). The plant material was oxidized with nitrobenzene in alkali and the resulting lignin degradation products—vanillin, syringaldehyde, and (where the quantity permitted) *p*-hydroxybenzaldehyde—were isolated by partition chromatography on a Celite column (2, 3).

Measurement of Radioactivity

All samples were counted as carbon dioxide in a gas phase counter (4). The radioactivity was calculated as microcuries by reference to a carbon-14 standard obtained from the Bureau of Standards, Washington, U.S.A.

Results and Discussion

Utilization of Tyrosine by Various Plant Species

In Part IV of this series (2) preliminary evidence was presented of marked species differences in the ability of plants to utilize tyrosine as a lignin precursor. Wheat plants, which are monocotyledons, were able to use tyrosine as efficiently as phenylalanine, which is one of the best lignin precursors yet studied. On the other hand, tyrosine was virtually inactive for lignin biosynthesis in the dicotyledons, maple, poplar, and caragana. The study has now been extended to seven additional species, so that a total of four monocotyledons and seven dicotyledons have been compared.

The figures for carbon-14 dilution* given in Table I show that the last four species, which are all dicotyledons, were not able to utilize tyrosine appreciably compared to phenylalanine. Neither were the two monocotyledons, *Carex* and *Smilacina*. A third monocotyledon, *Calamagrostis inexpansa*, on the other hand, resembled wheat in being able to convert both amino acids to lignin equally well. Both *Calamagrostis* and *Triticum* are members of the family Gramineae, and are the only members of this family yet investigated. The above data suggest that the ability to utilize tyrosine for lignin synthesis is comparatively restricted among the higher plants, being possessed by members of only one of the three families of monocotyledons and none of the species representing the six families of dicotyledons investigated.

It was suggestive that the Gramineae appear to be almost the only plants which yield appreciable amounts of *p*-hydroxybenzaldehyde on nitrobenzene oxidation. In view of this it was considered possible that, in families which cannot use tyrosine for lignin formation, ring substitution proceeds in such a way that no intermediate which is substituted *only* in the para position is involved. This situation would exist if the meta position were substituted first. To check this possibility *m*-methoxycinnamic acid was fed to both wheat and maple. The dilution of the carbon-14 relative to phenylalanine is shown in Tables II and III, respectively, and it is seen that *m*-methoxycinnamic acid was utilized very inefficiently by both species. This result argues against the first ring substitution being in the meta position, but on this evidence alone it is still possible that the unmethylated *m*-hydroxycinnamic acid could be an intermediate. Although the origin of the lignin methyl group has been thoroughly explored by Byerrum and co-workers (5), there is as yet little evidence as to the stage at which methylation occurs, and it cannot be assumed that it takes place before ring substitution is complete. However, further evidence against the above hypothesis is provided by the data for *p*-hydroxycinnamic acid. The fact that this compound is efficiently converted to at least two of the lignin residues by maple rules out the idea that this species is unable to metabolize *p*-hydroxylated C_6, C_3 substances in general.

It appears probable, therefore, that the distinctive metabolic pattern in the Gramineae pertains to the utilization of tyrosine only. A likely explanation is that grasses possess a tyrosine-metabolizing enzyme not present in the other families (or at least individual members) studied. The participation of phenylpyruvic acid in lignin biosynthesis has already been reported (2) and it is possible that the enzyme in question is a transaminase or oxidase which forms the analogous *p*-hydroxyphenylpyruvic acid. Alternatively, a block may exist beyond *p*-hydroxyphenylpyruvic acid, preventing its conversion to lignin monomers. This is speculation as yet, because the existence of such

**The phenylalanine and tyrosine used in these experiments were biologically synthesized and are assumed to be randomly labelled. As only seven-ninths of the carbon-14 in these molecules is actually recovered in the phenolic aldehydes from lignin, whereas all of it is recovered when specifically β-labelled precursors are administered (2), a correction has been applied in reporting the specific activities of the two amino acids. The values in the tables are seven-ninths of the measured values for the administered amino acid. This correction should be applied to the data in the preceding papers, although it is not large enough to change the conclusions therein.*

TABLE I

CONVERSION OF PHENYLALANINE AND TYROSINE TO LIGNIN IN SEVEN PLANT SPECIES

Species of plant	Compound	Dose, μM./gm. dry wt.	Specific activity of aldehydes from lignin, μc./mM.		Dilution* (Corr. for dry wt. of plant)	
			Vanillin	Syringaldehyde	Vanillin	Syringaldehyde
Carex laeviconica Dewey	P†	0.35	0.376	0.636	3700	2200
	T†	0.32	0.005	0.004	240,000	300,000
Calamagrostis inexpansa Gray	P	0.41	0.855	1.06	1640	1320
	T	0.35	0.454	0.664	2600	1780
Smilacina stellata (L.) Desf.	P	0.29	1.62	1.42	865	986
	T	0.30	0.016	‡	88,000	‡
Salix amygdaloides Anderss.	P	0.26	0.789	1.46	1780	959
	T	0.29	0.011	0.031	130,000	49,000
Melilotus officinalis (L.) Lam.	P	0.24	0.865	0.950	1620	1470
	T	0.26	0.010	0.010	160,000	160,000
Eleagnus commutata Bernh.	P	0.27	0.741	0.595	1890	2360
	T	0.28	0.008	0.005	170,000	280,000
Fagopyrum tataricum (L.) Gaertn.	P	0.27	1.44	1.18	972	1190
	T	0.35	0.005	0.006	360,000	300,000

**Observed dilution equals the specific activity of the administered compound divided by the values in columns 4 or 5.*
†P = L-phenylalanine and T = L-tyrosine. The specific activities were 1400 and 1360 μc./mM., respectively. The solutions were 0.0025 M.
‡Insufficient recovered for analysis. p-Hydroxybenzaldehyde was obtained from this species, but the chromatographic fraction was associated with relatively large amounts of oily impurities.

TABLE II

CONVERSION OF CINNAMIC ACID DERIVATIVES TO LIGNIN IN WHEAT

Compound	Specific activity, μc./mM.	Dose, μM./gm. dry wt.	Specific activity of aldehydes from lignin, μc./mM.			Dilution* (Corr. for dry wt. of plant)		
			Vanillin	Syringaldehyde	*p*-Hydroxybenzaldehyde	Vanillin	Syringaldehyde	*p*-Hydroxybenzaldehyde
L-Phenylalanine†	23.5	16.2	0.148	0.212	0.089	159	111	264
Phenylpropionic acid†	31.4	14.2	0.067	0.087	0.105	410	315	262
Caffeic acid†	39.6	15.9	0.228	0.180	0.016	171	216	2450
L-Phenylalanine‡	46.7	6.8	0.285	0.367	0.119	164	127	392
m-Methoxycinnamic acid‡	114	8.6	0.064	0.011	0.003	2100	12,000	45,000
p-Hydroxycinnamic acid‡	31.4	6.1	0.101	0.125	0.296	278	224	95
Ferulic acid‡	87.5	8.3	0.680	0.273	0.039	156	387	2700
Sinapic acid‡	63.8	7.4	0.001	0.444	0.022	70,000	156	3100

**Observed dilution equals the value in column 2 divided by that in columns 4, 5, or 6.*
†Experiment 1.
‡Experiment 2.
NOTE: *The solutions of active compounds were 0.053 M in experiment 1 and 0.027 M in experiment 2.*

TABLE III

CONVERSION OF CINNAMIC ACID DERIVATIVES TO LIGNIN IN MAPLE

Compound	Specific activity, μc./mM.	Dose, μM./gm. dry wt.	Specific activity of aldehydes from lignin, μc./mM.		Dilution* (Corr. for dry wt. of plant)	
			Vanillin	Syringaldehyde	Vanillin	Syringaldehyde
L-Phenylalanine	21.6	9.9	0.242	0.125	89	173
Cinnamic acid	31.4	12.6	0.255	0.050	157	805
p-Hydroxycinnamic acid	31.4	11.3	0.169	0.065	212	550
m-Methoxycinnamic acid	87.5	21.2	0.041	0.004	4500	47,000
Caffeic acid	39.6	12.8	0.290	0.117	177	440
Ferulic acid	30.8	13.8	0.456	0.106	95	406
Sinapic acid	31.4	12.9	0.055	0.875	745	47

**Observed dilution equals the value in column 2 divided by that in columns 4 or 5.*

NOTE: *The solutions of active compounds were 0.053 M.*

enzymes in higher plants is virtually an unexplored field. Wilson, King, and Burris (19) have, however, demonstrated a weak transamination from both tyrosine and phenylalanine to α-ketoglutaric acid in barley and lupine. More experimental work in this field is clearly indicated.

Formation of Lignins from Cinnamic Acid Derivatives

The data in Tables II and III were obtained from experiments conducted under similar conditions, and permit a comparison of the utilization of cinnamic acid derivatives in wheat and maple. Five β-labelled compounds of this type were administered to each species. A sixth, β-phenylpropionic (dihydrocinnamic) acid, was fed to wheat only, as it was not available when the experiments with maple were done. Cinnamic acid was administered only to maple in these experiments; a comparison of its metabolism in the two species may be obtained by reference to the earlier data for wheat (2), but it should be noted that the experiments were done at widely different times. As maple yields very small quantities of *p*-hydroxybenzaldehyde, a study of the formation of the corresponding lignin residue in maple was not feasible.

It can be seen from the tables that the two species are similar in the efficiency with which a given precursor is transformed to a given lignin residue, in relation to the standard lignin precursor, phenylalanine. The only marked difference is shown by sinapic acid, which is used to a much greater extent by maple than by wheat to form the guaiacyl lignin residue. However, the amount of sinapic acid used by maple for this purpose is still relatively unimportant. The dilutions of the C^{14} in *m*-methoxycinnamic acid are too high to have much quantitative significance.

Because of these similarities in lignin biogenesis between such unrelated species, it is probable that the metabolic pathways leading to the formation of lignins from labelled precursors are essentially similar in other species. However, differences may exist in the metabolism of individual compounds, as has been shown for tyrosine, and suggested (2) for cinnamic acid and vanillin. Thus, caution should be used in reasoning from one species to another.

Evidence obtained by Freudenberg and his associates would appear to support the mechanism proposed (9) for the polymerization of coniferyl alcohol, a monomer with unsaturation in the side chain, to guaiacyl lignin. In the present experiments β-phenylpropionic acid, the only C_6, C_3 compound yet studied which has a saturated, unsubstituted side chain, was converted to lignins nearly as efficiently as was phenylalanine. The fact that these two compounds and, in some species, tyrosine are so readily utilized raises the question whether they are first metabolized to monomers with unsaturated side-chains, or whether an alternative route to lignin involving polymerization of saturated monomers may also be available.

Preferential Synthesis of Lignins

It should be emphasized that there is normally considerable variation in the efficiency with which plants of the same species convert the same precursor to

different lignins. For example, when phenylalanine was fed to wheat at three different times but under similar conditions the V/S ratio (where V and S are the dilutions of C^{14} in vanillin and syringaldehyde, respectively) varied from 1.0 to 1.4, and in two experiments with maple an even greater difference, 0.5 to 1.4, was noted. Variations of this order in one experiment cannot, therefore, be taken as demonstrating preferential synthesis of one or another lignin from a labelled precursor. Within these limitations, though, several inferences can be drawn from a comparison of the transformation to lignins of precursors with varying degrees of ring substitution. On the basis of an earlier experiment with ferulic acid (2), in which this compound was preferentially converted into the analogous guaiacyl lignin, we expressed the belief that each type of lignin polymer has a corresponding monomer, i.e. that (meta) ring substitution precedes polymerization. Other evidence favoring this theory has been advanced recently by Higuchi *et al.* (11). These workers incubated embryonic root-tip tissue of kidney bean, and bamboo-shoot tissue, with compounds of the guaiacylpropane and syringylpropane type. When the tissues were oxidized with nitrobenzene, syringaldehyde was produced only from the tissues incubated with syringylpropane compounds; no evidence of formation of syringyl lignin from guaiacylpropane units was found.

We have suggested (2) that the synthetic route between the pool of C_6, C_3 metabolites and syringyl lignin likely involves sinapic acid. The marked preferential synthesis of syringyl lignin from sinapic acid in the present work, in both wheat and maple, supports this contention. The results of the current experiments with ferulic acid are less clear-cut, but taken together with those obtained previously they leave little doubt that this compound is used primarily for the synthesis of guaiacyl lignin and that, as might be anticipated, it is not used appreciably to form *p*-hydroxyphenyl lignin. The transformation of *p*-hydroxycinnamic acid to *p*-hydroxyphenyl lignin* could be studied only in wheat, and here the differences are not great enough to permit a final conclusion, although the figures suggest that this reaction is predominant. The unmethylated dihydroxy-compound, caffeic acid, is a good precursor of both guaiacyl and syringyl lignin in both species. In maple, at least, the figures indicate more efficient transformation to the guaiacyl residue but, as with *p*-hydroxycinnamic acid, one experiment is insufficient to allow more than tentative conclusions to be drawn.

It is to be expected that preferential conversion would be increasingly harder to demonstrate as precursors with less ring substitution are used, for the rates of the reactions leading to further ring substitution may at times approach that of polymerization and thus mask the effect. Variations in the relative rates of these competing reactions under different conditions may explain the different degrees of preferential synthesis of guaiacyl lignin from ferulic acid.

**Recent work by Smith (16) with wheat native lignin suggests the "p-hydroxyphenyl lignin" referred to here may consist of p-hydroxycinnamic acid ester groups.*

Acknowledgments

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REGIONAL DIFFERENCES IN THE GLUCAGON CONTENT OF PANCREAS FROM ALLOXANIZED DOGS[1]

By Sergio A. Bencosme and S. Mariz

Abstract

The uncinate process of the dog pancreas is devoid of A cells. Pancreatic extracts from the uncinate process of alloxanized dogs showed neither hyper- nor hypo-glycemic activity. These findings suggest that the lack of hyperglycemic effect of extracts from the uncinate process of the normal dog pancreas (11) simply indicates absence of glucagon and is unrelated to the presence of insulin. These results are in agreement with the hypothesis that glucagon is originated by the A cells of the pancreas. Neither the D nor X cells showed any sign of damage following alloxan injection during the period of observation.

A clear correlation has been established between the content of glucagon in the dog pancreas and the presence or absence of A cells. Pancreatic extracts from the uncinate process normally devoid of A cells showed a lack of hyperglycemic effect in contrast with the marked hyperglycemic effect of extracts from the body and tail which contain A cells (6). Since the presence of insulin in those extracts might have obscured a small hyperglycemic effect, and because preliminary studies with cysteine did not prove in our hands to be a successful method for separating the insulin activity from that of the glucagon in extracts from the uncinate process, it was decided to reinvestigate, on a regional basis, the glucagon content of the pancreas in the alloxanized dog. The results of the present work confirm the fact that the uncinate process of the dog pancreas is devoid of A cells and any detectable amount of glucagon, as measured by the qualitative method used.

Material and Method

Eight mongrel dogs of both sexes, weighing between 21 and 35 lb., received 60 mgm./kgm. of alloxan monohydrate (Eastman Kodak) in a 3% solution by a single intravenous injection in the leg veins. Animals were killed with an overdose of Nembutal as follows: five at two days, and one each at three, five, and eight days after alloxan injection. In all cases, the uncinate process was removed for glucagon extraction immediately after death, a section adjacent to the body being used for histological study. With this procedure, one can invariably know after microscopic examination whether A cells are present distal to the point of biopsy (5). Representative areas from the body of the pancreas of these alloxanized dogs were used for control purposes and therefore glucagon extraction and histological examination were also carried out in some of them (Fig. 1). Pancreatic tissues used for glucagon extraction were each immediately placed, separately, in acid alcohol and were then minced. Extracts prepared by the method of Best *et al.* (7) were dried and

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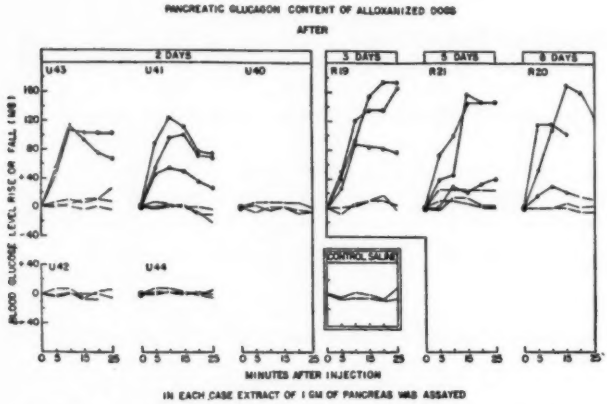

FIG. 1. Represents blood sugar changes expressed as milligrams per cent (mgm.) in cats when injected with extracts from body of pancreas (– – –) or from the uncinate process (–·–·–) of alloxanized dogs.

kept in the refrigerator until ready for use. The tissues studied histologically were fixed in Zenker formol solution and stained with the aldehyde fuchsin (8) and with the author's modifications of the Masson's trichrome and Gomori chrome alum haematoxylin (1). For estimating the glucagon content, the dried extracts were dissolved in physiologic saline (0.9% sodium chloride) acidified to pH 2.5 with hydrochloric acid. Two cubic centimeters of solvent was used for each gram of pancreas. The procedure suggested by Staub and Behrens (10) was followed. After an overnight fast, normal cats weighing between 2.5 and 3.5 kgm. received 80 mgm./kgm. of sodium Amytal. The inguinal veins were exposed bilaterally and duplicate blood samples were obtained just prior to and at 5, 10, 20, and 25 min. after the injection of the dissolved extract into the contralateral vein. The blood glucose was determined by micromodification of the Folin-Wu method (9). Extracts of 1 gm. of pancreatic tissue from each of the various portions were tested (Fig. 1). Unless the amount of extracts available from the uncinate process was insufficient, these extracts were tested in triplicate. For control purposes, 2 cc. of the acidified physiologic saline used to dissolve the pancreatic extracts was tested (Fig. 1).

Results

Histological sections of the uncinate process did not reveal the presence of A cells, except on one occasion, (R19) in which the biopsy was obviously taken just at the borderline between the uncinate process and the body of the pancreas, thereby including a few pancreatic lobules with A cells. A cells were, on the other hand, numerous and apparently undamaged in the body of the pancreas of all animals.

A few occasional well-granulated beta cells were seen in the uncinate process of the alloxan treated dogs. Only once (U41) did we find a somewhat larger number of degenerating beta cells with pycnotic nuclei but still with well-stained granules. On the other hand, in the body of the pancreas of dogs killed two days after the injection of alloxan, some degenerating beta cells with pycnotic nuclei were still present. The pancreatic islets of animals killed three days after alloxan injection were comprised almost exclusively of A and D cells. Exceedingly few well-granulated B cells were seen after this period. In no instance did we observe any alteration of the D or X cells in the pancreas of the alloxanized dogs.

The changes in blood sugar levels of the fasting, anesthetized cats after the injection of each extract, or of the acidified physiologic saline, are illustrated in Fig. 1. As was to be expected, the portion of the pancreas containing A cells uniformly elicited an initial marked hyperglycemic response. On the other hand, except for extracts from R21, which produced some hyperglycemic effect, and extracts from U41, which produced minimal hypoglycemic effect, all the other extracts from the uncinate process of these alloxanized dogs induced curves which were essentially similar to those obtained when the acidified physiological saline was tested.

Discussion

The results of these experiments demonstrated an almost complete lack of effect of extracts from the uncinate process of the alloxanized dog upon the blood sugar of the cat, whereas extracts from the body of the dog pancreas invariably showed a marked hyperglycemic effect in all tested cases. The assay procedure is not specific for either glucagon or insulin, but the blood sugar changes induced in the cats may be taken to indicate the end result of the combined action of insulin and glucagon when these are present together. B cells were largely absent from the sections of alloxanized dogs' pancreas, and it is well known that when most B cells are destroyed by alloxan, pancreatic insulin content becomes profoundly reduced (11). It is reasonable to suppose, therefore, that the effect of our extracts on blood sugar levels is related principally to the concentration of glucagon, uninfluenced by possible differences in insulin content.

At present, we can offer no definite explanation for the mild hyperglycemic activity of the extract from the uncinate process of R21. It may be explained on the basis that there was some contamination from the body of the pancreas, or that some amount of the hyperglycemic factor is normally present in the uncinate process which, on some occasions, may be enough to be detected by the qualitative method used here.

We take our results to indicate that the lack of hyperglycemic effect from the extracts of the uncinate process of the dog pancreas is not related to the presence of insulin, but rather to the lack of sufficient glucagon.

The present work strongly supports the idea that glucagon originates from the A cells. This is in agreement with our previous findings in untreated

dogs (6) as well as with those obtained in cobalt-treated guinea pigs (4). In the latter experiments, almost complete destruction of A cells was necessary before pancreatic extracts were devoid of a hyperglycemic effect to the same extent as extracts from the A cell-free uncinate process of untreated dogs. On the other hand, pancreatic extracts from guinea pigs, in which up to about 90% of their A cells were completely degranulated or vacuolated, gave curves indistinguishable from those of the untreated control guinea pigs.

Another interesting aspect of this work is the fact that the recently described X cells of the dog pancreas (5), particularly abundant in the uncinate process, were not affected by alloxan administration, nor did they show any evidence of transformation into either A or B cells. Similarly, no sign of damage to the D cells could be established. The lack of alteration of both D and X cells was in agreement with the findings reported earlier on the cytogenesis of islet cells and on the cytology of alloxan diabetes in the rabbit in which it was postulated that the A, B, and D cells were independent islet cells from their origin and remained so during life (2). It was also shown that most agranular islet cells of alloxan diabetic animals are D cells and that these were not clearly affected at any time during the course of the alloxan diabetes (3). These findings suggest that the X cell is also an independent islet cell like the A, B, and D cells, and that neither the D nor X cell participates in the formation of insulin.

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FURTHER STUDIES ON THE METABOLISM OF UROCANIC-α-C^{14} ACID[1]

By M. Kraml and L. P. Bouthillier

Abstract

The existence of radioactive metabolites in the liver extracts of rats a short time after injection of urocanic-α-C^{14} acid has been investigated. Glutamic acid, N-formiminoglutamic acid, and an unknown compound that yields glutamic acid on hydrolysis were the major urocanic acid catabolites found therein. These three substances accounted for as much as 75% of the total radiocarbon in the extracts. The sole catabolic pathway of importance for urocanic acid, in the rat, is the formation of glutamic acid. Since formylglutamic acid could not be detected in these extracts, the role of this substance as an obligatory urocanic acid catabolite becomes questionable.

Introduction

In a previous paper we presented evidence that glutamic acid is an important metabolite in the pathway of urocanic acid degradation (8). We now wish to report the results of subsequent experiments which show that the glutamic acid pathway is the major pathway of urocanic acid dissimilation in the intact rat. Evidence is also presented to show that N-formiminoglutamic acid is an intermediate in the sequence of metabolic reactions.

Experimental*

Materials

The method of synthesis of the urocanic-α-C^{14} acid used in our experiments has been described previously (8). The specific activity of the material was 1.15×10^6 counts per minute per milligram. L-Glutamic acid upon treatment with 88% formic acid in the presence of acetic anhydride yielded N-formyl-L-glutamic acid, m.p. 109°–10°. Calculated for $C_6H_9NO_5$, N : 8.00. Found N : 7.93. N-Formyl-γ-benzyl-L-glutamate was amidated using the ethyl-chlorocarbonate method (2, 3). Catalytic hydrogenation of the resulting amide afforded N-formyl-L-isoglutamine, m.p. 130°–32°. Calculated for $C_6H_{10}N_2O_4$, N : 16.08. Found N : 16.01. The synthesis of L-isoglutamine was described in a previous paper (9). N-Formimino-L-glutamic acid was prepared by the method of Miller and Waelsch (11). Accordingly γ-benzyl-L-glutamate was condensed with formamidine hydrochloride (4) in the presence of silver carbonate to yield γ-benzyl-N-formimino-L-glutamate, m.p. 169°–70° (dec.). Calculated for $C_{13}H_{16}N_2O_4$, N : 10.60. Found N : 10.21. Hydrogenation of this compound (in 50% methanol) at atmospheric pressure, in the presence of platinum oxide catalyst, afforded N-formimino-L-glutamic acid. The presence of the glutamic acid derivative in the solution was ascertained

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Contribution from the Department of Biochemistry, Faculty of Medicine, University of Montreal, Montreal, Que.
**All melting points were taken by the capillary method and are uncorrected.*

by the following tests, in accordance with those already reported: (*a*) a positive but slow reaction with ninhydrin (10), (*b*) a characteristic pink spot on paper chromatograms after treatment with the ferricyanide–nitroprusside reagent (7), and (*c*) an R_f value of 0.56 in *tert*-butanol : formic acid : water (14 : 3 : 3) (18) and of 0.42 in *sec*-butanol : formic acid : water (19 : 2 : 6) (10).

Biological Methods

Male Wistar strain rats, weighing between 40 and 50 gm. each, were injected intraperitoneally with about 10 mgm. of urocanic-α-C^{14} acid dissolved in 1.5 ml. of distilled water (pH adjusted to 7.4 with sodium hydroxide). Rats 1 and 2 were sacrificed 15 min. after injection, Rats 3 and 4 after 30 min., and Rat 5 after 60 min. The entire liver of each animal was immediately removed, ground with a Potter homogenizer containing 10 ml. of distilled water, and the homogenate was deproteinized with 10 ml. of 20% trichloroacetic acid (TCA). The denatured protein was filtered off, the filtrate was extracted with ether to remove the TCA, and the volume was made to 25 ml. with water.

Chromatography

The liver extracts were assayed for total radiocarbon content and also analyzed for the presence of metabolites by paper and column chromatography. Radioactive peaks on the paper chromatograms were detected by assaying the eluates of segments cut from the chromatograms (8). Identification of a particular metabolite was obtained by co-chromatography with known substances. Amino acids and isoglutamine were detected on chromatograms, using the ninhydrin reaction. N-Formiminoglutamic acid was characterized by the ferricyanide–nitroprusside reagent. Formylglutamic acid and formylisoglutamine were revealed by the starch – potassium iodide – chlorine method of Rydon and Smith (14). Samples of liver extracts of Rats 1 and 2 were refluxed in 6 *N* hydrochloric acid for four hours and the hydrolyzates were then chromatographed. The radioactivity then found as glutamic acid is referred to in Table I as "Total glutamic acid". The disappearance of any radioactive peak accompanied by a corresponding increase in the glutamic acid peak would identify the metabolite as an intermediate in the urocanate–glutamate conversion. A 5 ml. aliquot of Rat 1 liver extract was lyophilized and the residue was taken up in 1 ml. of 1 *N* hydrochloric acid. The solution was chromatographed on a 1 × 50 cm. column of Dowex-50-X8 cation exchange resin (200–400 mesh). Hydrochloric acid (1, 2, and 4 *N*) was used as the eluting agent (16). Volumes of 2.5 ml. were collected using a volumetric fraction collector. Aliquots of each fraction were evaporated in Tygon-painted stainless steel cups and the radioactivity was measured.

Results

Incorporation and Distribution of Radiocarbon in Liver Extracts

The radioactive metabolites present in the liver extract of Rat 1 were separated by paper chromatography, and the distribution of the radioactivity is presented in Fig. 1 (A). When the same extract was analyzed by chromatog-

TABLE I

DISTRIBUTION OF RADIOACTIVITY ON CHROMATOGRAMS OF LIVER EXTRACTS OF RATS INJECTED WITH UROCANIC-α-C¹⁴ ACID

Rat No.	Duration of experiment, min.	Total C¹⁴ on the chromatograms, %						C¹⁴ content of liver extracts, as % of the dose injected
		Peak I, glutamic acid	Peak II, unknown	Peak III, N-formimino-glutamic acid	Peak IV, unknown	Peak V, urocanic acid	Total glutamic acid*	
1	15	18	37	21	8	6	65	17
2	15	8	11	61	13	2	75	14
3	30	18	39	18	14	0	—	6
4	30	14	23	—	43	0	—	9
5	60	14	34	0	31	0	—	4

**After refluxing aliquots of liver extracts for four hours in 6 N hydrochloric acid.*

raphy on a column of Dowex-50-X8 ion exchange resin, using hydrochloric acid as eluant, a comparable distribution was obtained (Fig. 2). There are five main radioactive peaks present in the extract. However, the more sensitive technique of column chromatography revealed the presence of other radioactive substances of minor importance not detected by paper chromatography. Peak I was identified as glutamic acid by the order of emergence from the Dowex-50 column and then by admixture chromatography on paper. Peaks II and III are glutamic acid containing metabolites for they were converted to glutamic acid when subjected to hydrolysis in 6 *N* hydrochloric acid. Peak IV is unknown while peak V is due to unchanged urocanic-α-C^{14} acid. The substance at peak V gave a positive Pauly-diazo test for imidazoles

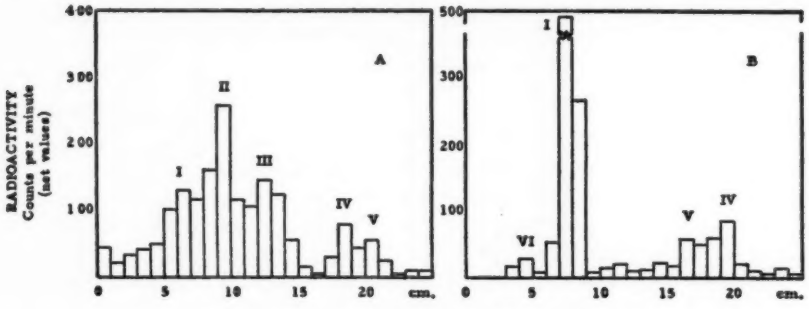

FIG. 1. Paper chromatography of liver extract.

(A) Rat 1 sacrificed 15 min. after injection of urocanic-α-C^{14} acid. Solvent system employed: phenol saturated with borate buffer, pH 9.3. Peak I is glutamic acid; III, N-formiminoglutamic acid; V, urocanic acid; while II and IV are unknown.

(B) Acid hydrolyzate (6 *N* HCl) of same extract chromatographed in phenol : water (4 : 1). Peaks II and III have almost entirely disappeared while peak I (glutamic acid) increased correspondingly. Peaks IV and V remained unchanged, their relative positions being reversed in this solvent. Peak VI is aspartic acid.

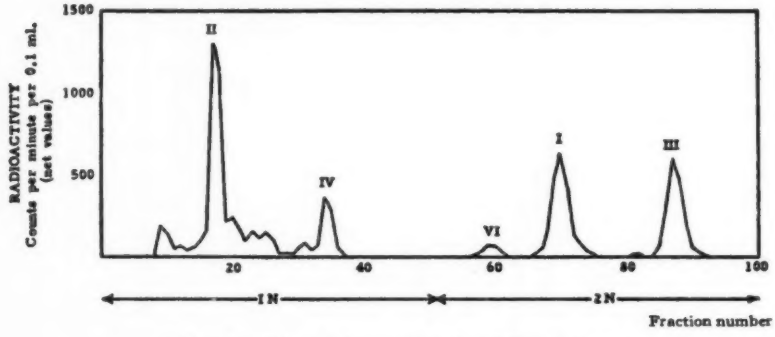

FIG. 2. Column chromatography of liver extract.

Rat 1 sacrificed 15 min. after injection of radioactive urocanic acid. 50 × 1 cm. column of Dowex-50-X8 resin. 1, 2, and 4 *N* HCl used as eluant. Peak I is glutamic acid; III, N-formiminoglutamic acid; VI, aspartic acid; II and IV are unidentified. Urocanic acid (not shown) was eluted from the column with 4 *N* HCl.

and was identical with authentic urocanic acid by co-chromatography. The results obtained with the other rats are summarized in Table I. The maximum incorporation of radiocarbon into the liver extracts, about 15%, was obtained with Rats 1 and 2, sacrificed 15 min. after injection. As the duration of the experiment was prolonged all peaks diminished, with the exception of peak IV. Urocanic acid was absent from the extracts 30 min. after injection while its metabolic derivatives, including glutamic acid, were still present in large amounts. Glutamic acid and its precursors (measured as the sum of peaks I, II, and III, or as glutamic acid after hydrolysis) accounted for about 75% of the C^{14} present in the liver extracts of Rats 1 and 2.

Identification of Peak III as N-Formiminoglutamic Acid

In Fig. 1 (B) is given the distribution of radioactivity on a typical paper chromatogram of acid hydrolyzed liver extract of Rat 1. The large peak was due to glutamic acid. Peaks II and III had almost completely disappeared, whereas IV and V remained the same (their relative positions being reversed in the solvent system employed). The increase in radioactivity of the glutamic acid peak paralleled very closely the disappearance of the metabolites at peaks II and III. The latter two substances must of necessity contain in their molecule the elements of glutamic acid. Attempts were thus made to identify these metabolites by comparing them with the synthetic intermediates prepared by us. Peak III proved to be due to N-formiminoglutamic acid. Admixture chromatography (Table II) in seven solvent systems showed that the radioactivity of peak III coincided in each instance with the pink spot of synthetic N-formimino-L-glutamic acid. It was also found that when larger amounts of the extracts were chromatographed, the compound at peak III gave the characteristic pink spot when treated with the ferricyanide – nitroprusside reagent (7). In spite of the fact that peak II also yielded glutamic acid on hydrolysis, we were unable to identify this peak as any one of our synthetic metabolites. It failed to coincide chromatographically with isoglutamine, formylisoglutamine, or formylglutamic acid.

Discussion

Isotopic studies have shown that both histidine (1, 20) and urocanic acid (8) are converted to a significant extent to glutamic acid in intact animals. More recent studies (3, 12, 17), carried out *in vitro*, have pointed out that both compounds are degraded to similar catabolites. The evidence strongly favored urocanic acid as a key intermediate in L-histidine degradation. However, the most serious objection to a major role for urocanic acid came as a result of the finding of Celander and Berg (5) that L-histidine is glycogenic whereas urocanic acid is not. Hence it would appear that even though urocanic acid may be degraded to glutamic acid (8), the extent of the reaction might be limited. However, the results reported in this paper show that as much as 75% of the radiocarbon present in the deproteinized liver extracts of rats (sacrificed 15 min. after injection with urocanic acid) is under the form of glutamic acid

TABLE II

IDENTIFICATION OF PEAK III AS N-FORMIMINOGLUTAMIC ACID

Solvent	R_f values*				
	Peak III	N-Formimino-L-glutamic acid	L-Isoglutamine	N-Formyl-L-glutamic acid	N-Formyl-L-isoglutamine
Phenol saturated with borate buffer, pH 9.3	0.50	0.52	—	—	—
Phenol : water (4 : 1)	0.54	0.53	—	—	—
n-Butanol : glacial acetic acid : water (15 : 3 : 7)	0.22	0.22	—	0.62	0.46
Benzene : *n*-butanol : methanol : water (1 : 1 : 2 : 1)	0.34	0.35	0.27	—	0.44
sec-Butanol : formic acid : water (19 : 2 : 6)	0.42	0.42	0.22	0.66	—
tert-Butanol : formic acid : water (14 : 3 : 3)	0.58	0.56	0.36	0.71	—
n-Propanol : 0.1 *N* acetic acid (3 : 1)	0.28†	0.28	—	—	0.44

**The R_f values of the standard substances were obtained after the substances were mixed with an aliquot of liver extract prior to ascending paper chromatography. Values for the latter three compounds are given only for the solvents in which their R_f values differed markedly from that of the radioactive metabolite.*

†*In this solvent, both the radioactive metabolite and N-formimino-L-glutamic acid had a tendency to show a double spot, the lower one having an R_f value of 0.14 in each case.*

and its precursors. The same extracts contained about 5% of unchanged urocanic acid. It is evident that the conversion of urocanic acid to glutamic acid did occur rapidly and to a very large extent, so large in fact that the existence of any other important route of urocanic acid dissimilation becomes extremely doubtful. This finding coupled with those reported by other authors now firmly establish urocanic acid as the prime intermediate in the glycogenic metabolism of L-histidine. As we mentioned in our previous paper, the non-glycogenic behavior of urocanic acid may be explained on the basis that injected urocanic acid is readily eliminated (6), thus preventing significant glycogen deposition. On the other hand, urocanic acid normally formed from L-histidine in the liver cells would just as rapidly be catabolized; urocanic acid would not accumulate and hence there would be no urinary excretion.

The extracts were found to contain two radioactive metabolites that yielded glutamic acid on acid hydrolysis. The compound at peak II could not be identified as any one of the glutamic acid derivatives prepared by us. The possibility remains that this unidentified substance might be a cyclic precursor of N-formimino-L-glutamic acid, perhaps imidazolonepropionic acid. However, peak III corresponded to N-formiminoglutamic acid. This compound has previously been recognized as an intermediate product of histidine degradation *in vitro*, in the presence of cat (3) and guinea pig liver extracts (18), and also in some bacterial extracts (10, 18, 19). We would like to point out that formylglutamic acid was not found in our extracts even though large amounts of glutamic acid were formed from urocanic acid. Knudson (7), who reported that fortified liver homogenates could degrade labeled N-formiminoglutamic acid, also failed to detect formylglutamic acid in the incubation media, upon chromatographic analysis. While it has been established that N-formyl-L-glutamic acid is an intermediate in histidine metabolism for certain bacteria (17, 18), other workers have found that N-formimino-L-glutamic acid is converted directly to glutamic acid and formamide by *Aerobacter aerogenes* (10) and by *Clostridium tetanomorphum* (19). A similar observation was reported to the effect that formylglycine is not an intermediate in the dissimilation of N-formiminoglycine by *Clostridium acidi-urici* (15). The repeated failure to find a radioactive peak corresponding to formylglutamic acid, after the degradation of either urocanic acid or N-formiminoglutamic acid to glutamic acid, raises the question as to whether this compound is in reality an obligatory intermediate in histidine metabolism by mammalian tissue. Its role remains to be definitely established.

Now with regard to the possible role played by isoglutamine, Abrams and Borsook (1) reported that, on the basis of preliminary evidence, this substance was an intermediate in histidine catabolism in mammalian tissue. In our extracts we failed to find any evidence for the presence of isoglutamine and/or formylisoglutamine.

Certain Japanese workers (12, 13) were able to isolate, from bacterial digests of histidine, substances whose analyses and chemical properties resembled closely those of formylisoglutamine and isoglutamine. They

claimed that glutamate formation from histidine, or urocanic acid, in bacterial extracts involved these compounds and that the opening of the ring of imidazolonepropionic acid resulted in the formation of formylisoglutamine. To explain histidine dissimilation in certain bacteria they proposed a scheme involving these two compounds. Histidine catabolism in mammalian tissue, however, does not appear to follow this route.

Acknowledgment

The authors are indebted to the 'Fondation J. Rhéaume' for supporting this research.

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HEMODYNAMIC CHANGES FOLLOWING AORTIC CONSTRICTION IN NORMAL AND IN HYPOPHYSECTOMIZED RATS[1]

By Margaret Beznák

Abstract

Systolic and diastolic blood pressures were measured electrically, during ether anesthesia, in the carotid and femoral arteries at different times after subdiaphragmatic aortic constriction in normal and hypophysectomized rats. In normal rats there is a rise in both systolic and diastolic pressures above the stricture, followed by a transitory drop, then a gradual increase to hypertensive levels. Below the constriction the pressure falls sharply with a great reduction in pulse pressure, but rises to values above normal in a few days. In hypophysectomized rats the initial changes parallel those in normal rats, but the rise of the blood pressure to hypertensive levels both above and below the stricture is absent. Left ventricular hypertrophy develops from about the second day on in normal but not in hypophysectomized rats. After constriction there is a small decrease in heart rate followed by a pronounced tachycardia in normal but not in hypophysectomized rats. There is some indication of an increased subdiaphragmatic peripheral resistance in hypophysectomized rats.

Introduction

Aortic constriction causes an increase in heart weight and blood pressure in intact but not in hypophysectomized rats (8, 9). The changes in heart weight and blood pressure, measured at times varying from immediately after aortic constriction to five days later, occurring in normal rats were described earlier (5). Similar observations have now been made in hypophysectomized rats.

Methods

Male albino rats, Wistar strain, weighing 155–230 gm. were used. They lived in individual cages and received Master Fox cubes and tap water ad libitum. All operations were performed under ether anesthesia. Five days after hypophysectomy the aorta of the rats was constricted just below the diaphragm with a silver ring of 0.74 mm. diameter as previously described (2). Normal rats with similar aortic constriction were also included, because measurement of systolic and diastolic pressures was now possible while in earlier experiments only mean pressure could be measured. Hypophysectomized and normal rats were divided into eight groups with at least six rats each. The first group contained controls, without aortic constriction. In the second group the silver ring was put around the aorta but was closed only after the basal blood pressure was recorded. In the next groups of rats the blood pressure was recorded one, three, and six hours, one, two, and five days after the aorta was constricted. The blood pressure was measured electrically using the Hathaway Instrument, under ether anesthesia. The

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right femoral vein was cannulated and the rats given through it 0.3 ml. of a heparin solution containing 10 mgm. commercial heparin per ml. The carotid was cannulated with a $\frac{3}{4}$-in.-long No. 21 hypodermic needle and the femoral artery with a No. 25 hypodermic needle cut to a length of 5 mm. The needles were directly fitted on the pressure heads so as to avoid long fluid columns. The records obtained in the carotid represent the systolic–diastolic pressures with only minimal distortion, because cannulating the carotid in a few cases with a wider needle did not change the form of the record. The femoral records are probably damped but the extent of this could not be determined, because no needle wider than No. 25 or shorter than 5 mm. could be introduced into the femoral artery. The heart rate and finally the wet and dry weights of the heart and its different parts as well as some of the endocrines were determined.

Observations

Table I gives the systolic and diastolic blood pressures in the carotid and femoral arteries of normal rats and in rats that were hypophysectomized five days previously.

Fig. 1 shows the blood pressure changes taking place in normal and hypophysectomized rats at different times after aortic constriction. That the starting (before constriction) values are not exactly the same as those given in Table I is due to the fact that in the figure only the six rats of group 2 are considered. Thus values before aortic constriction and those immediately following it are measured in the same rats.

In normal rats the carotid pressure (systolic and diastolic) rises at first after constriction, then there is a fall of pressure followed by a gradual increase. There is a sharp fall in the femoral pressure immediately after constriction then from about a day after constriction it rises steadily to above the normal value. The femoral pulse pressure shows a great decrease immediately after constriction and only rises gradually. The lower part of the figure shows the blood pressure measurements at the same intervals in hypophysectomized rats. It is apparent from this figure that changes in the carotid pressure during the first one to two days following aortic constriction are similar to

TABLE I

SYSTOLIC AND DIASTOLIC PRESSURE IN THE CAROTID AND FEMORAL ARTERIES OF NORMAL AND HYPOPHYSECTOMIZED RATS

		Carotid		Femoral	
	n	Systolic	Diastolic	Systolic	Diastolic
Normal	21	134 ± 3	92 ± 4	130 ± 4	95 ± 3
Hyptomy*	22	92 ± 4	60 ± 4	94 ± 5	73 ± 4

NOTE: n = *number of rats. For the pressure values the average ± the standard error of the mean are given.*

**Five days after hypophysectomy.*

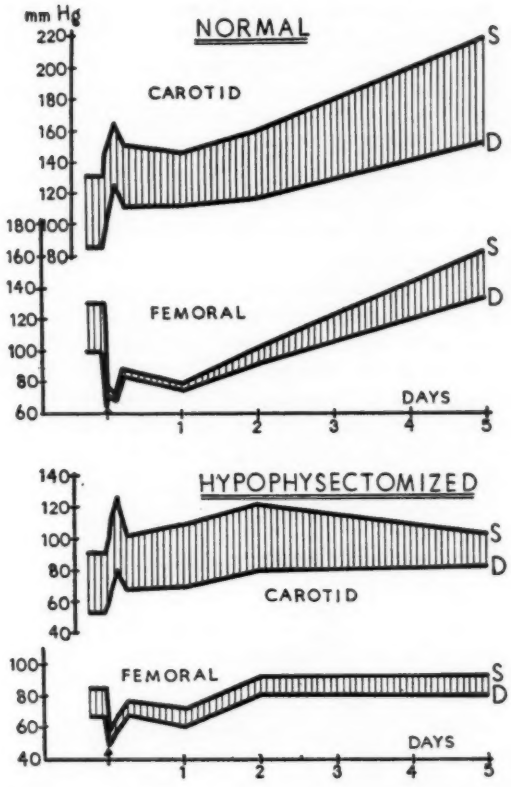

FIG. 1. Changes in carotid and femoral blood pressures following subdiaphragmatic aortic constriction in normal (upper part) and hypophysectomized rats (lower part). Ordinates: Blood pressure in mm.Hg. Abscissae: Days after aortic constriction. The upper lines of the carotid and femoral pressures represent the systolic (*S*), the lower lines the diastolic (*D*) pressures. The shaded areas thus correspond to pulse pressure. The time of constriction is marked by the arrow.

those observed in normal rats. After that, however, the carotid pressure does not show the steady rise that is seen in normal rats. Another difference is the decrease in the carotid pulse pressure five days after aortic constriction. At this time systolic, diastolic, and pulse pressures are all increased in normal rats. In hypophysectomized rats, on the other hand, while the diastolic pressure is some 30 mm. higher than it was before constriction, the systolic pressure is only 10 mm. higher. As to the femoral pressure, the hypophysectomized animals also exhibit a sharp initial fall of systolic, diastolic, and pulse pressures seen in normal rats, but the subsequent rise in systolic and diastolic values does not significantly surpass the preconstriction level.

The changes in heart rate are plotted in Fig. 2. In normal rats and especially in hypophysectomized rats, there is a slight slowing of the heart after constriction of the aorta. Following this a highly significant tachycardia develops in normal rats one day after aortic constriction which is, however, absent in hypophysectomized rats. Five days after constriction the heart beats again with its preconstriction frequency in normal rats, but it is definitely slower in hypophysectomized rats.

As was discussed elsewhere (4), the pressure in the femoral artery after subdiaphragmatic aortic constriction depends essentially on the amount of blood entering through the constriction (thus on aortic, viz. carotid pressure) and on peripheral resistance in the lower half of the body (R_S). Let P_C and P_F be the mean aortic (or carotid) and femoral pressures. Then assuming that: (*a*) blood flow through the constriction approximately follows Poiseuille's law, which seems probable for so narrow an orifice, (*b*) blood viscosity and (*c*) effective dimensions of the stenosis remain constant, (*d*) venous pressure is not far from zero, and (*e*) femoral pressure is above the range for critical closing of smaller vessels, then the rate at which blood enters the abdominal aorta $Q_E = k\,(P_C - P_F)$, and the rate at which blood leaves the abdominal aorta $Q_L = P_F/R_S$. Since $Q_E = Q_L$, $R_S = P_F/k\,(P_C - P_F)$. These calculated

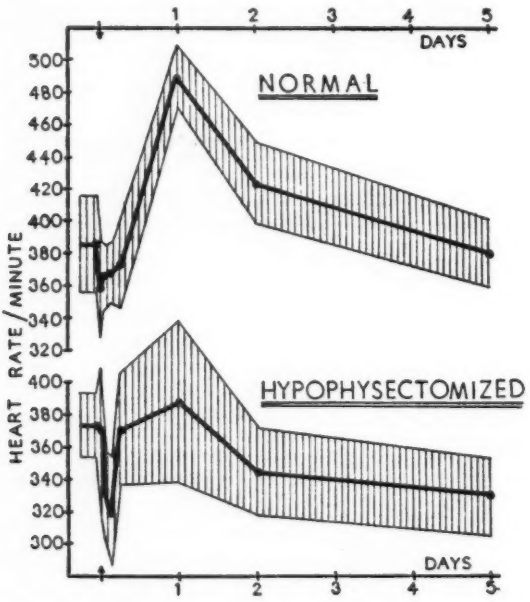

FIG. 2. Changes in heart rate following subdiaphragmatic aortic constriction in normal (upper part) and hypophysectomized rats (lower part). Ordinates: Heart rate per minute. Abscissae: Days after constriction. Shaded areas represent ± twice the standard error of the mean. The time of constriction is marked by the arrow.

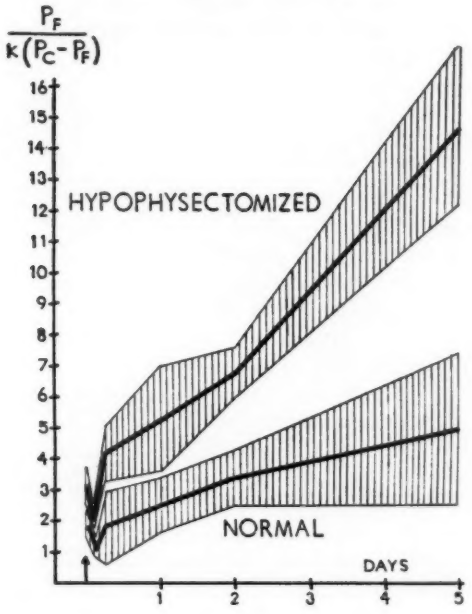

FIG. 3. Changes in $P_F/k\ (P_C - P_F)$ (for explanation see text) following aortic constriction in normal and hypophysectomized rats. Ordinate: Values for above expression. Abscissa: Days after aortic constriction. The time of constriction is marked by the arrow. Shaded areas represent ± twice the standard error of the mean.

values for R_S are plotted at different times after constriction in Fig. 3. Two points are worth noting in connection with this figure: first, that—as already described (5)—there is a small fall in subdiaphragmatic peripheral resistance during the first hours after aortic constriction, followed by a gradual rise; second, that changes are similar in hypophysectomized rats but the values of R_S are in all cases higher than in normal rats.

Concomitant changes in left ventricular weight are plotted in Fig. 4. Instead of using the left ventricle weight to body weight ratio, which also varies with the latter (6, 3), the expected left ventricle weight was calculated from an equation obtained elsewhere (5). The difference between the actually observed and the calculated weight was then expressed as percentage of the latter and this is plotted on the ordinate. The upper part of the figure shows that in normal rats after some initial oscillations—the significance of which is doubtful—the weight of the left ventricle increases and is 32% over the expected value by the fifth day. The lower part of the figure shows changes in left ventricular weight in hypophysectomized rats, at the same intervals after aortic constriction. It is interesting that the weight of the left ventricle increases during the first two days to almost exactly the same extent as it does in normal rats. After this, however, not only is there no further increase

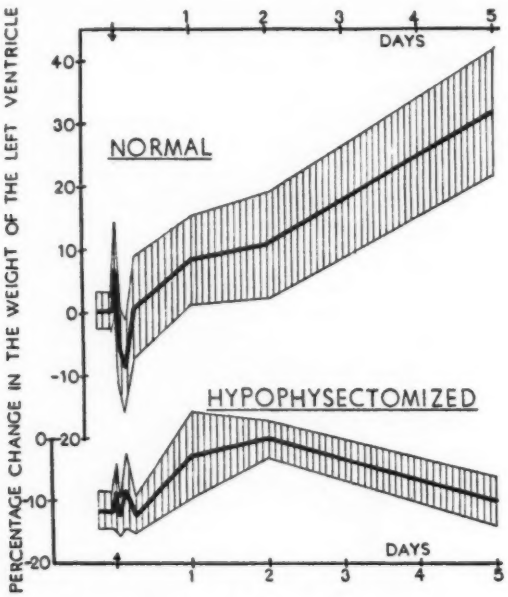

FIG. 4. Changes in the weight of the left ventricles following subdiaphragmatic aortic constriction in normal (upper part) and hypophysectomized rats (lower part). For details of calculating changes in left ventricular weight see text. Ordinate: Weight changes in %. Abscissae: Days after aortic constriction. Shaded areas represent ± twice the standard error of the mean. Time of constriction is marked by an arrow.

but the left ventricle actually loses the weight gained and its weight on the fifth day is the same as it was before the aorta was constricted. Attempts to correlate these changes with changes in water content were unsuccessful, the ratio of dry weight to water content of the hearts remaining unaltered at all times during these experiments.

Discussion

Constriction of the aorta is a mechanical hindrance and as such results in an increased pressure above the stricture and a fall below it. This takes place equally in normal and in hypophysectomized rats. It is very likely that the increase in pressure above the stricture stimulates the pressoreceptors. This is indicated by the slight slowing of the heart and by the decrease in subdiaphragmatic peripheral resistance. It seems that after some time the factors that enable the heart to expel blood against the suddenly increased resistance become exhausted and the carotid pressure starts to fall. This may reduce the discharge rate of the pressoreceptors which had become accommodated to the existing higher blood pressure and thus cause the increase in heart rate. In hypophysectomized rats the increase of the heart rate does

not take place. This seems to indicate that it is about this time that the heart of hypophysectomized rats starts to differ from that of normal rats. After the period of tachycardiac compensation cardiac hypertrophy develops in normal rats. At the same time the heart rate gradually falls back to the normal level, and the blood pressure rises. In hypophysectomized rats there is no cardiac hypertrophy and no hypertension, only a gradual slowing of the heart.

Fig. 3 showed that the subdiaphragmatic peripheral resistance (R_S) after aortic constriction is greater in hypophysectomized than in normal rats. Calculation of R_S in the manner described is valid only if the dimension of the stenosis remains constant. Development of an effective collateral arterial circulation would tend to raise the pressure in the lower half of the body and would mimic an increase in peripheral resistance. It may be that the late apparent rise in R_S is due to the growth of such a collateral pathway. The higher values of R_S in hypophysectomized rats may, therefore, be due to an increased vascular tone or to a more effective development of a collateral circulation. The former seems to be more likely for two reasons: (1) The R_S in the lower half of the body is higher in hypophysectomized rats at all times after constriction, i.e. also immediately after it, when no collateral circulation could as yet have developed. (2) Five days after aortic constriction the diastolic pressure in the carotid (above the constriction) is 30 mm.Hg higher than it was before constriction while the systolic pressure is only 10 mm.Hg higher.

It seems then that the initial changes following aortic constriction are similar in normal and in hypophysectomized rats and are the mechanical consequence of the stricture. If in rats an increased systolic output adds to the higher pressure above the constriction as Gupta and Wiggers have shown for dogs (7), then this seems to happen also after hypophysectomy. After a day or two, however, hypophysectomized rats fail to show the cardiovascular changes observed in normal rats. There are two main possible explanations for this difference: (1) If the changes following aortic constriction are independent of the kidneys as Alexander believes (1), the failure may be due to the lack of some pituitary hormones acting directly on heart and blood vessels. Our experiments provide some indirect evidence to show that the failure occurring in hypophysectomized rats around the first to second day after aortic constriction may indeed be primarily cardiac. (*a*) The subdiaphragmatic peripheral resistance is higher in hypophysectomized than in normal rats. (*b*) In hypophysectomized rats five days after constriction the diastolic pressure in the carotid is much higher than before constriction, while the systolic pressure is only slightly elevated. This indicates that the absence of hypertension a few days after aortic constriction in hypophysectomized rats is not due to a failure on the part of the small vessels to constrict and thus maintain a resistance. Rather, the resistance being there, the heart—unable to exert force—gradually fails to maintain the blood pressure. (2) Rytand's experiments (12, 13), on the other hand, point to the

involvement of the kidneys in the hypertension developing after aortic constriction. Kohlstaedt and Page (11) and Hawthorne, Perry, and Pogue (10) have shown that it is the reduction in pulse pressure that stimulates the production of renal pressor substances. In our experiments a very great reduction in femoral pulse pressure took place after aortic constriction, in accordance with the results of Sealy (14), Gupta and Wiggers (7), and Alexander (1). A comparable fall in femoral pulse pressure, however, also occurred in our hypophysectomized rats. This indicates that the stimulus for the production of pressor substances is present in hypophysectomized rats, but the production of pressor substances is either absent or reduced after hypophysectomy (for a discussion of this problem see Wakerlin (15)). Finally it is also possible that though these substances are produced, the hypophysectomized heart cannot respond to them, as outlined above.

Acknowledgment

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HEPARINASE

III. PREPARATION AND PROPERTIES OF THE ENZYME[1]

By M. H. Cho[2] and L. B. Jaques

Abstract

The extraction of heparinase from beef and rabbit liver has been studied. Optimal conditions for precipitation of the enzyme were 33% ammonium sulphate, pH 7.0; 7% ethanol, pH 5.0–5.5; 40–70% acetone, pH 8.0; with 0.1% NaCl for ethanol and acetone. A new method of preparation of the enzyme is described, using extraction with 0.15 *M* KCl, precipitation with 33% ammonium sulphate, isoelectric precipitation at 6.0, elution at 37° C., and precipitation at 5.0. This preparation gave a 50% destruction of heparin in three hours. The optimum pH was 4.8, substrate concentration, 0.2 mgm./ml. Higher concentrations inhibited the enzyme. A linear relation was not obtained between enzyme concentration and velocity of the reaction. The enzyme was inhibited by mercuric chloride, magnesium chloride, lithium chloride, iodoacetate, glutathione, methionine, versene, and citrate. In liver homogenates, the enzyme activity was associated with all fractions (microsomes, mitochondria, etc.). Active preparations were obtained from liver of man, ox, pig, rabbit, guinea pig, rat, gopher; kidney of ox, pig, rabbit, guinea pig, and rat; muscle of rabbit and guinea pig. No activity was obtained from dog liver, ox testis and thymus, human placenta. Difficulties in assaying heparinase in tissue extracts are discussed.

The enzyme heparinase was first demonstrated by Jaques (3) using extracts of rabbit liver. Monkhouse and Jaques (9) developed a simple method for the extraction of heparin from blood using phenol, and this was applied by Jaques and Keeri-Szanto (6) to extraction of heparin in the enzyme–substrate system. They reported that the antithrombin activity of heparin and metachromatic activity of the heparin disappeared in a parallel fashion. Thus the action of heparinase could be followed fairly easily by extraction of the enzyme–substrate with phenol and measurement of the metachromatic activity of the heparin remaining. This method has now been applied to studies of the preparation and properties of heparinase in greater detail.

Methods

Estimation of Heparin

This was based on measurement of the metachromatic activity of heparin as described by Jaques, Bruce-Mitford, and Ricker (5). The method was adapted to the Coleman Jr. Spectrophotometer, and the increased light absorption of the dye azure A at wave length 500 mμ when heparin was added was determined. Azure A (National Aniline Division, Allied Chemical and Dye Corp., certified by the Biological Stain Commission) was prepared as a stock solution of 100 mgm.% (w/v) and stored in the refrigerator. This stock solution was diluted 1 : 10 for use. A borate buffer was prepared by dissolving 19.1 gm. of sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) and 12.4 gm. of boric acid

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[2]*Canadian Life Insurance Medical Research Fellow, University of Saskatchewan.*

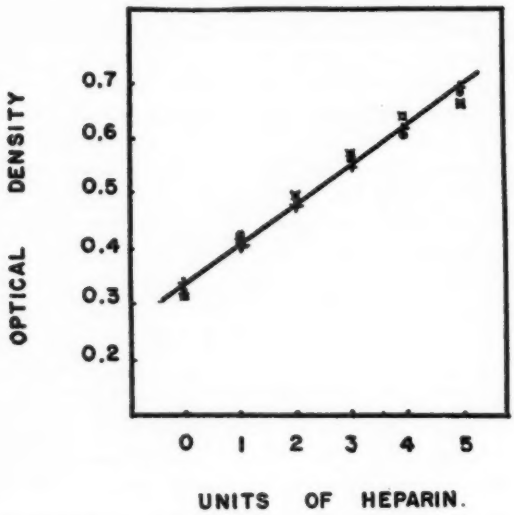


FIG. 1. Metachromatic assay of heparin. Optical density of 2.5 mgm. % azure A at 500 mμ and pH 8.3 measured in Coleman Jr. Spectrophotometer.

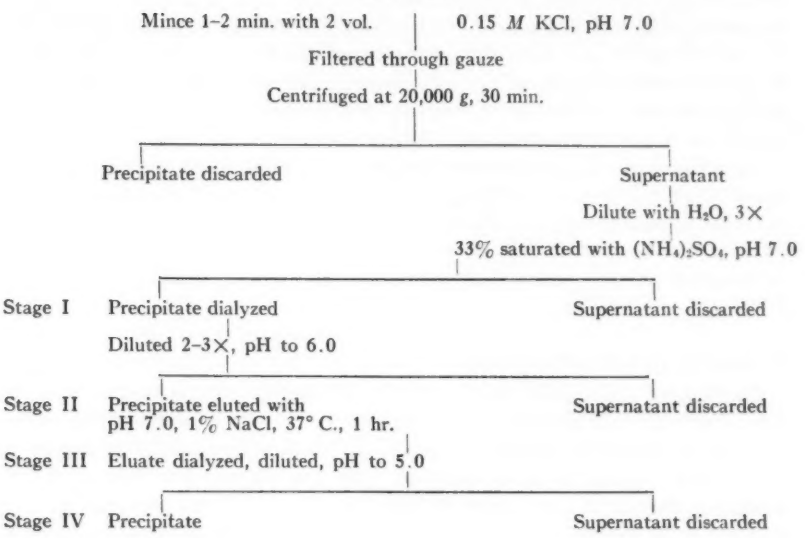
(H_3BO_3) in 2 liters of distilled water, and its pH was adjusted to 8.3 using *N* NaOH or *N* HCl. Usually 0.3 ml. of sample solution was added to 2.7 ml. of borate buffer, 1 ml. of 10 mgm. % azure A dye was added just before the measurement, mixed well, and the increased absorption of light measured. The amount of heparin was calculated from a standard curve prepared by adding known amounts of standard heparin to the borate buffer solution. Distilled water was used for the blank setting of the instrument. Fig. 1 shows the standard curve for the estimation of metachromatic activity of heparin using the method described above.

Preparation of Crude Enzyme Extract

The method of Jaques (3) with slight modifications was used for the preparation of crude extracts of heparinase. The tissue was minced with two volumes of glycerol in a Waring Blendor for two to three minutes and dialyzed against running tap water for 48 hr. If the temperature of the tap water was above 15° C., the dialysis was carried out against distilled water in the cold room (4–6° C.). The dialyzed extract was centrifuged to remove precipitated material and the clear supernatant was then lyophilized. This material was stored for up to a month at 4–6° C. without loss of activity. When reconstituted with water or phosphate–acetate buffer, this gave a crude extract used for the fractionation experiments. At first rabbit liver was used for the extraction of heparinase. However, once enzyme activity had been demonstrated in beef liver (Table VII), beef liver was used owing to the greater availability of this tissue.

TABLE I

FLOW CHART OF METHOD FOR PREPARATION OF HEPARINASE



Method of Preparation of Heparinase

A new method for the preparation of heparinase was developed and is summarized in Table I. The entire process was carried out at a temperature below 6° C., except the incubation at 37° C. Fresh ox liver was frozen and stored in the deep freeze. The partially thawed and cut tissue was minced with two volumes of 0.15 *M* KCl solution at pH 7.0 for two minutes in a Waring Blendor. The homogenate was then passed through gauze to remove the coarse material and the turbid extract was centrifuged for 30 min. at 20,000 *g* in a Serval angle centrifuge to remove the minute tissue particles. The supernatant was diluted with one volume of distilled water, and saturated $(NH_4)_2SO_4$ solution at pH 7.0 added to 33% saturation. After it had been allowed to stand more than three hours, the precipitate formed was removed by centrifugation (15 min., 5000 *g*) and dialyzed against running tap water for three to four hours (if the water temperature was below 15° C.) and then against distilled water for 12–15 hr. with constant stirring until free of $(NH_4)_2SO_4$ in the dialyzate, as tested by $BaCl_2$ solution. The dialyzate was diluted three times with distilled water, the pH adjusted to 6.0 with dilute acetic acid. After more than six hours, the precipitate (pH 6 precipitate) was collected by centrifugation (15 min., 3000 *g*). This pH 6 precipitate was mixed with appropriate amounts of 1% NaCl solution at pH 7.0 and warmed in a 37° C. water bath with constant mechanical stirring. After one hour

incubation, the precipitate was removed by centrifugation (20 min., 5000 *g*) and the NaCl extract was dialyzed against distilled water for six hours. When a white precipitate started to appear inside the cellophane sac, the contents was diluted three times with distilled water, the pH adjusted to 5.0, and the mixture left to stand about six hours. The fine white precipitate was collected by centrifugation (15 min. 3000 *g*) and suspended in an appropriate amount of 0.15 *M* acetate buffer at pH 5.0. Only a part of it dissolved in the acetate buffer solution. When this enzyme preparation was stored in the cold (0–1° C.) it retained its activity for about one week.

In preparing a large quantity of enzyme, it was convenient to collect and store individual batches at Stage I. After dialysis the pH was adjusted to 6.0 and the suspension stored in the deep freeze. When a sufficient number of batches had been collected, they were reconstituted by adding two to three volumes of distilled water and collecting the precipitate after six hours for Stage II.

Estimation of Enzyme Activity

Connaught Laboratory beef heparin, lot No. 1196, was used. This was the sodium salt and labelled as 96 units/mgm. A stock solution of 1 mgm./ml. of heparin in 0.15 *M* acetate buffer at pH 5.0 was prepared and kept in the refrigerator. Prior to an experiment, this was diluted five times with the acetate buffer and 0.5 ml. portions taken in 125 × 15 mm. test tubes. The tubes were brought to 37° C., and 0.5 ml. of enzyme solution (at pH 5.0), also at 37° C., was added. In the early stages of the investigation, 5.0 ml. of enzyme was incubated with 5.0 ml. of heparin and 1 ml. of the mixture was pipetted out for assay. The enzyme assay for the heparinase was then conducted at pH 6.0 with acetate–phosphate buffer, since the pH optimum for the crude enzyme lies in this range (6). However, better preparations of heparinase showed a pH optimum at 5.0 and therefore the studies of enzyme properties were conducted at this pH.

After the reaction mixture was incubated for the desired number of hours in the 37° C. water bath with constant agitation of the tubes, the reaction was stopped by addition of 1 ml. of 80% phenol and coagulated protein was separated by centrifugation (15 min. 3000 *g*). The supernatant aqueous layer which contained heparin was removed, dissolved phenol extracted with ether, and the ether removed with warming at 60° C. for 30 min. When the aqueous layer was cooled, 0.3 ml. was added to 2.7 ml. of borate buffer and the amount of heparin present measured. Assays were run in duplicate, and controls containing enzyme alone and heparin alone were also included in the experiment.

With crude heparinase extracts, it required 12 hr. incubation to destroy 30–50% of the substrate. This was the difference in the value for extractable heparin between experimental and control tubes. Control tubes showed anywhere from 70% loss of heparin, caused by protein binding, to 50–100% increase caused by extraction of substances affecting heparin metachromasia.

A marked *decrease* in the value for heparin in the control tubes could be seen with heparinase prepared by 33–50% saturation of ammonium sulphate at pH 7.0 from beef liver extract. It was especially marked with the preparation obtained at 50–70% saturation. When the control and experimental tube both showed the loss of heparin in a parallel fashion the heparinase preparation was considered inactive and was discarded. A marked *increase* in the value for the heparin in the control tubes could be seen both with active enzyme preparations and inactive preparations. The active preparation showed the decrease of heparin content in the experimental tubes while the inactive preparation showed no change. The pH 6.0 precipitate in the final method described above showed little or no increase of control values of heparin usually but when it was further purified to the Stage IV of Table I, an increased value of metachromasia in the control of anywhere from 0 to 50% could be seen, while the experimental value showed a decrease of metachromasia of more than 30% in three hours' incubation. In the assay of the early crude heparinase preparations, the difference between the values obtained for the control and experimental tubes was taken as representing the action of the enzyme. In the evaluation of more purified enzyme preparations, the actual decrease of the experimental heparin value from zero time to the time of measurement was alone considered and the increased value of the control was ignored. The reason for this will be discussed later.

Results

Fractionation Tests of the Crude Enzyme

The new method for the preparation of heparinase is based on the results of fractionation tests of crude heparinase reported here. Optimum salt and solvent concentration and optimum pH were determined for the fractionation of crude heparinase with ammonium sulphate, cold acetone, and ethanol. Ammonium sulphate fractionation tests were carried out in the cold room at 4–6° C. The fractionation using acetone and ethanol was done at −7° C., since at room temperature these solvents inactivated heparinase. Both rabbit and beef liver were used. Unless otherwise stated, 2–3 gm. of each lyophilized preparation was dissolved in 200 ml. of distilled water or 0.1 *M* acetate–phosphate buffer used for each fractionation.

Fractionation of Heparinase with Ammonium Sulphate

Beef liver crude extract was precipitated with ammonium sulphate at different pH values ranging from 3 to 8. Ten milliliter aliquots of crude extract were adjusted to the desired pH with either 0.1 *N* NaOH or 0.1 *N* acetic acid. Saturated ammonium sulphate solution was adjusted for the different pH values using concentrated H_2SO_4 or NH_4OH and 30 ml. of this solution was then added to the aliquots of the crude liver extract at the same pH. The precipitates were centrifuged down, dialyzed, and tested for enzyme activity. The upper part of Table II shows the results of these experiments which indicate that the optimum value for the precipitation of heparinase with ammonium sulphate is pH 7.0.

TABLE II

PRECIPITATION OF HEPARINASE WITH AMMONIUM SULPHATE

A. Crude liver extract after adjustment of pH was precipitated in 10 ml. portions with 30 ml. of saturated ammonium sulphate at the same pH. The precipitate was dialyzed, made up to 10 ml. and 5 ml. incubated with 5 ml. of heparin (0.2 mgm./ml.) for 12 hr. at 37° C., with 5 ml. as control. Results are shown in μgm. of commercial heparin destroyed.

pH	3.0	4.0	5.0	6.0	7.0	8.0
Heparin decrease (μgm.)	20	60	80	150	320	90

B. 200 ml. of crude liver extract was precipitated with 400 ml. of saturated ammonium sulphate solution. To the supernatant was added 280 ml. of saturated ammonium sulphate solution and to the further supernatant solid ammonium sulphate was added to the maximum saturation. After dialysis, the volume was made up to 11 ml. An aliquot was used to determine the dry weight and 5 ml. portions were used for experiment and control as in A.

	Beef liver			Rabbit liver		
% of $(NH_4)_2SO_4$	33	54	70	33	54	70
Total dry weight (mgm.)	990	1163	130	1400	805	765
Heparin decrease (μgm.)	390	120	60	90	360	30

Beef liver extracts were fractionated by the addition of increasing amounts of saturated ammonium sulphate solution at pH 7.0. As shown in the lower part of Table II most of the enzyme activity was precipitated with the first 33% saturation of ammonium sulphate. Rabbit liver gave similar results in most cases. However, sometimes it showed rather inconsistent results since the activity was occasionally associated with the 50% fraction or even with the 70% fraction of ammonium sulphate saturation as shown in Table II. Beef liver crude extract showed more uniform results.

The Precipitation of Heparinase With Ethanol

The cold ethanol fractionation method for plasma protein described by Cohn (1) was applied to the fractionation of beef and rabbit liver. The crude extract was taken in 15 ml. aliquots and adjusted to pH values ranging from 4.5 to 7 with 10 ml. of 0.1 *M* acetate buffer. Cold ethanol (15 ml.) was added slowly to these aliquots, using a capillary pipette, while the mixture was stirred. The temperature of the mixture was always kept below −2° C., the precipitate was centrifuged down, washed with ethanol and ether, and tested for heparinase activity. Ionic strength was maintained at 0.1% NaCl throughout the fractionation. As shown in Table IIIA similar results were given by both beef and rabbit liver extracts with the optimum precipitation of the enzyme at 5.0-5.5.

The fractionation of the enzyme on the addition of increasing amounts of cold ethanol was tested at pH 5.5 and 0.2% NaCl concentration, to find out the optimum concentration of ethanol for the recovery of the enzyme. Using

TABLE III

PRECIPITATION OF HEPARINASE WITH ETHANOL

A. Crude liver extract after adjustment of pH was precipitated in 25 ml. portions with 15 ml. of ethanol. The precipitate was washed with ethanol and ether and dissolved in 11 ml. of pH 6.0, 0.1 *M* acetate–phosphate buffer. 5.0 ml. of this preparation was incubated with 5 ml. of heparin (0.2 mgm./ml.) for 12 hr. at 37° C., with 5.0 ml. as control. Results shown in μgm. of heparin destroyed.

pH		4.5	5.0	5.5	6.0	6.5	7.0
Heparin decrease (μgm.)	Rabbit	27	46	42	32	35	17
	Beef	10	50	52	45	32	10

B. 200 ml. of crude extract at pH 5.5 was precipitated with successive addition of ethanol i.e. 15, 30, 50, 100, and 200 ml. and each fraction was collected and tested for heparin as in A.

% of ethanol	7	13	20	30	50
Total dry weight (mgm.)	296	273	82	208	269
Heparin decrease (μgm.)	106	0	0	27	61

C. For 25 ml. aliquots of crude liver extract after adjustment of the pH to 5.5, desired amount of solid NaCl was added to make the final concentration as listed in the table and then 25 ml. of ethanol. The precipitate was tested for heparinase activity as in A.

% of NaCl	0.2	0.4	0.6	0.8	1.0	2.0
Heparin decrease (μgm.)	32	24	25	15	22	10

200 ml. of beef liver extract five fractions were collected. As shown in Table IIIB, it was found that the initial fraction with 7% ethanol and the last two fractions with 30 and 50% ethanol contained the most activity.

By varying the NaCl concentration from 0–2% at pH 5.5, the effect of ionic strength for the precipitation of heparinase with ethanol was tested using 25 ml. aliquots of crude extract and 50% ethanol. A part of the results are reported in Table IIIC. Identical recoveries were obtained with 0, 0.05, 0.10, 0.15, and 0.2% NaCl.

The Precipitation of Heparinase with Acetone

Heparinase was fractionated with acetone at the pH range of 6 to 8 and salt concentrations of 0, 0.1, and 0.2% NaCl. For each precipitation, a 325 mgm. aliquot of crude extract was dissolved in 10 ml. of 0.1 *M* phosphate buffer of desired pH and 30 ml. of acetone was added at the different levels of pH and final salt concentration. The results are shown in Table IVA and it can be seen that a pH value of 8.0 and 0.1% NaCl gave the best yield of the enzyme. The use of pH values higher than 8.5 was not desirable since the enzyme was inactivated rapidly about that pH. Table IVB shows the results of the experiments in which increasing concentrations of acetone were added to a partially purified crude extract (50% $(NH_4)_2SO_4$ saturation) and

TABLE IV

PRECIPITATION OF HEPARINASE WITH ACETONE

A. 10 ml. aliquot of crude extract at the desired pH in 0.1 *M* phosphate buffer was precipitated with 30 ml. of acetone. The precipitate was dissolved in 11 ml. of pH 6.0 acetate–phosphate buffer. 5.0 ml. of this preparation was incubated with 5 ml. of heparin (0.2 mgm./ml.) for 12 hr. at 37° C., with 5 ml. as control. Results shown in μgm. of commercial heparin destroyed.

% NaCl	pH		
	6.0	7.0	8.0
0	26	75	160
0.1	0	0	490
0.2	45	100	25

B. 200 ml. of initial extract at pH 8.0 was precipitated with the addition of increasing amounts of acetone and each precipitation was collected and tested for heparinase as in A. Starting material used was (*a*), (*b*) partially purified by 50% saturation of $(NH_4)_2SO_4$, (*c*) a fresh tissue extract after it was minced and clarified by centrifugation.

		% of acetone concentration				
		15	30	40	70	75
(*a*)	Total dry weight (mgm.)	67	354	336	501	521
	Heparin decrease (μgm.)	0	55	110	215	107
(*b*)	Total dry weight (mgm.)	70	305	327	571	530
	Heparin decrease (μgm.)	10	5	50	375	45
(*c*)	Total dry weight (mgm.)		332	1181	634	
	Heparin decrease (μgm.)		33	118	63	

to a fresh tissue extract (liver minced with water and clarified by centrifugation). The initial volume in each case was 200 ml., the NaCl concentration was 0.1%, and the pH was 8.0. Some difference was observed between the partially purified liver extract and the fresh liver extract in the manner of fractionation of heparinase with acetone. In the former case, precipitation of activity occurred at the concentration of 40–70% but in the latter case, the heparinase activity precipitated at the 30% and the 70% concentration of acetone.

From the above experiments, and other observations, the heparinase seems to be associated with the globulin fraction of extracts from beef liver. Since beef liver gives much more consistent results than rabbit liver, it was used exclusively in further study.

Properties of Heparinase

With active enzyme preparation, prepared by the new method described above (Table I, Stage IV), some properties of the enzyme were studied. Unless otherwise stated the reaction mixture was the same as described under Methods.

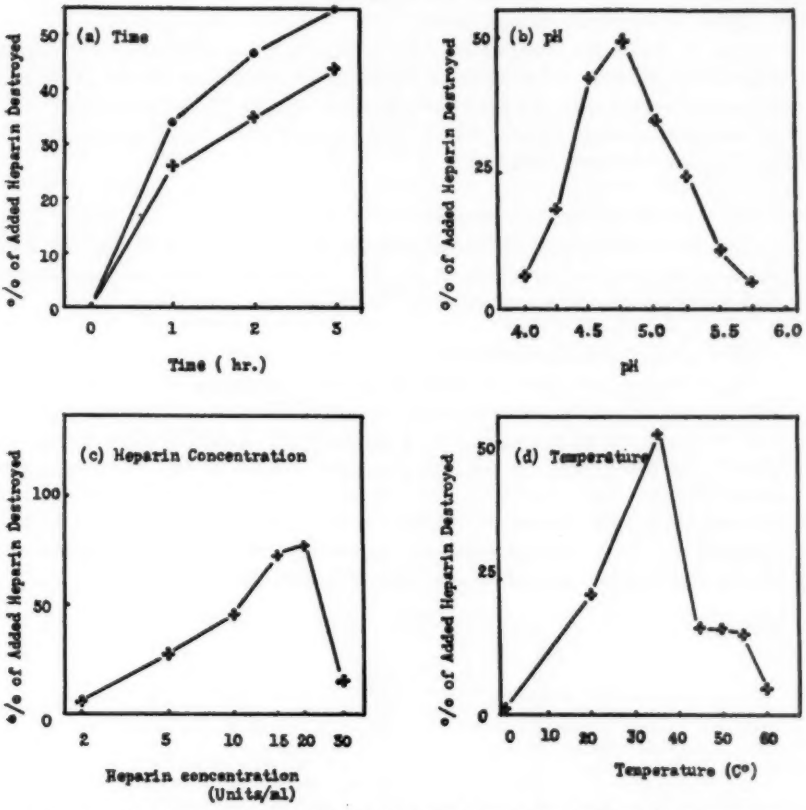


FIG. 2. The effect of (*a*) time, (*b*) pH, (*c*) heparin concentration, and (*d*) temperature on the per cent of added heparin destroyed in three hours by heparinase from beef liver ·—· and rabbit liver +—+. Enzyme, Stage IV preparation in 0.5 ml. of 0.15 *M* acetate buffer added to 0.5 ml. of heparin in buffer. pH 5.0 in (*a*), (*c*), and (*d*), and adjusted to value shown in (*b*). Temperature 37° C. in (*a*), (*b*), and (*c*), and the value shown for (*d*). Final concentration of commercial beef heparin in the system was 0.1 mgm./ml. in (*a*), (*b*), and (*d*), and as shown in (*c*).

Effect of Time of Incubation

Fig. 2*a* shows the effect of time of incubation on the destruction of substrate heparin by heparinase, under optimal conditions of pH and substrate concentration. The amount of substrate destroyed increased with the time of incubation up to three hours, and a gradual decrease in the reaction velocity during the latter part of incubation was often noticed.

Effect of pH

The effect of pH was determined over the range of 4.0–7.0 in 1.15 *M* acetate buffer at a final substrate concentration of 0.1 mgm./ml. heparin. The results are shown in Fig. 2*b*. It is evident that the pH optimum for heparinase is 4.8 in 0.15 *M* acetate buffer with beef heparin as substrate.

Effect of Substrate Concentration

Fig. 2*c* shows the effect of varying substrate concentration on the reaction velocity at pH 5.0. The optimum substrate concentration for the heparinase–heparin reaction with the given preparation is approximately 20 units per ml. of heparin (0.2 mgm./ml.). Above this concentration very marked inhibition by the substrate occurred.

Effect of Incubation Temperature

The effect of temperature on heparinase activity is shown in Fig. 2*d*, which shows a temperature optimum of 37° C. With three hours of incubation at higher temperatures, inactivation of the heparinase occurred.

Effect of Enzyme Concentration

Fig. 3 shows the effect of varying enzyme concentration on the reaction velocity under optimal conditions of pH and substrate concentration (pH 5.0 and 15 units/ml. of heparin). It is immediately apparent that the relative activity of the enzyme is not directly proportional to the relative enzyme concentration and therefore the linear relationship obtained between concentration and velocity of reaction with most enzymes does not hold for heparinase. This unusual situation is probably due to the fact established above that heparin is itself an inhibitor of heparinase.

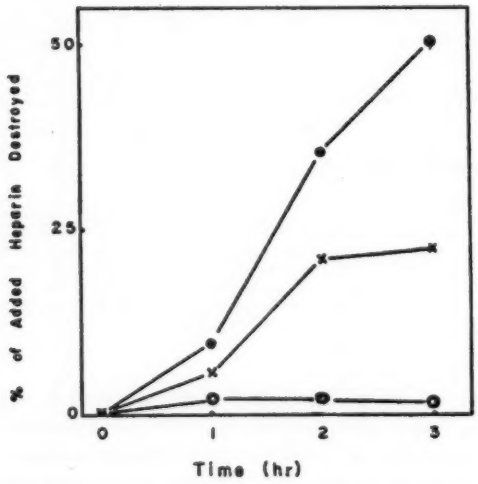


FIG. 3. The effect of enzyme concentration on the rate of destruction of heparin by heparinase.

Enzyme, Stage IV preparation, 0.5 ml. in 0.15 *M* acetate buffer pH 5.0 was added to 0.5 ml. of 0.3 mgm. heparin/ml. in the same buffer and incubated at 37° C. for zero, one, two, and three hours.

●——● original enzyme solution.
×——× fivefold dilution of the original enzyme solution.
○——○ 10-fold dilution of original enzyme solution.

Enzyme Inhibitors

Using a Stage IV preparation, we have tested a few metals, SH reagents, and other electrolytes for their possible action on this enzyme. Some of the results are shown in Table V. A final concentration of 0.01 *M* of mercuric chloride, magnesium chloride, lithium chloride, and reagents such as iodoacetate, oxidized glutathione, and methionine caused considerable inhibition. It is interesting to note that sodium citrate and the chelating agent versene inhibited while calcium chloride increased heparinase activity.

TABLE V

THE ACTION OF SEVERAL REAGENTS ON HEPARINASE ACTIVITY

Reagents	Final molarity	Relative activity
Control	H_2O	100
$HgCl_2$	0.01	0
$MgCl_2$	0.01	83
LiCl	0.01	59
$CaCl_2$*	0.01	146
CH_2ICOOH	0.01	74
Methionine	0.01	66
Glutathione	0.01	46
Versene	0.01	61
Sodium citrate	0.1	19

**With crude enzyme, pH 6 precipitate.*

NOTE: *Enzyme–substrate mixture consisted of 0.4 ml. Stage IV enzyme in 0.15 M acetate buffer at pH 5.0, 0.1 ml. of reagent solution tested (at pH 5.0), and 0.5 ml. of 0.2 mgm./ml. heparin in 0.15 M acetate buffer at pH 5.0. The mixture was incubated at 37° C. for three hours. Relative activity refers to the ratio of heparin destroyed in three hours with added reagent to heparin destroyed in the corresponding control.*

Distribution of Heparinase

To investigate the location of the enzyme at the cellular level, a few experiments were carried out using the cell fractionation method with 0.88 *M* and 0.25 *M* sucrose of Hogeboom, Schneider, and Pallade (2). Heparinase activity was estimated with dialyzed extracts of nuclear mitochondria and microsome fractions. These fractions were suspended in appropriate amounts of pH 6 acetate–phosphate buffer and tested on heparin as is shown in Table VI. Heparinase of rabbit and rat liver is associated with all fractions of the cell and not particularly confined to any one fraction.

The organs of various animal species were tested for heparinase activity. The tissue was extracted in each case by 0.15 *M* KCl at pH 7.0 and precipitated with 50% saturation of ammonium sulphate. The precipitate was dialyzed and tested on heparin. The results are shown semiquantitatively in Table VII. The liver of man, ox, pig, rabbit, guinea pig, rat, and gopher showed heparinase activity but no activity was obtained from dog liver. The kidney of ox, pig, rabbit, guinea pig, and rat also showed heparinase activity.

TABLE VI

RELATIVE HEPARINASE ACTIVITY OF LIVER CELL FRACTIONS PREPARED BY CELL FRACTIONATION METHOD BY CENTRIFUGATION

Species	Rat liver	Rabbit liver	Rabbit liver
Sucrose solution	0.88 *M*	0.25 *M*	0.88 *M*
Fraction 1 cell debris	0.82	0.90	0.87
Fraction 2 mitochondria	0.41	0.60	0.32
Fraction 3 microsome and supernatant	0.36	0.73	2.07

NOTE: *Dialyzed cell fraction, 0.5 ml. at pH 6.0, was added to 0.5 ml. of 0.2 mgm./ml. heparin in pH 6.0 acetate–phosphate buffer and digested for three hours at 37° C. The amount of heparin destroyed in micrograms was divided by the dry weight of 1 ml. of cell fraction used and expressed as the relative activity.*

TABLE VII

SPECIES AND ORGANS TESTED FOR HEPARINASE

	Liver	Kidney	Muscle	Thymus	Testis	Intestine	Placenta	Lung
Man	+						−	
Ox	+	+		−	−			±?
Pig	+	+						
Rabbit	+	+	+			−		±?
Guinea pig	+	+	+			±?		
Rat	+	+						±?
Gopher	+							
Dog	−							

NOTE: *Tissue was minced with two volumes of 0.15 M KCl solution at pH 7.0 and precipitated with equal amount of saturated ammonium sulphate solution. The precipitate was dialyzed and appropriate amount was diluted with pH 6 acetate–phosphate buffer and incubated with heparin at 37° C. for 12 hr.* (+) *shows enzyme action,* (−) *indicates negative results, and* (±) *shows the results not consistent.*

Rabbit and guinea pig muscle were tested and both were positive. The testis of ox, which is a rich source of hyaluronidase, had no heparinase activity nor did ox thymus or human placenta. Rabbit and guinea pig intestine and ox, rabbit, and rat lung were tested with doubtful results.

Discussion

The assay of the enzymatic reaction of heparinase with heparin has presented a number of difficulties. As pointed out in the first paper on heparinase (3), heparin may be taken up by proteins in tissue extracts and thus removed non-enzymatically. This effect was distinguished from the enzymatic action by measuring the amount of heparin which disappeared in controls in which heparin and extract were mixed after incubation. It is not possible to use boiled enzyme for controls with heparin, since this increases the affinity of protein for heparin. It was hoped that the use of the phenol procedure and less crude enzyme preparations would obviate the difficulties encountered in

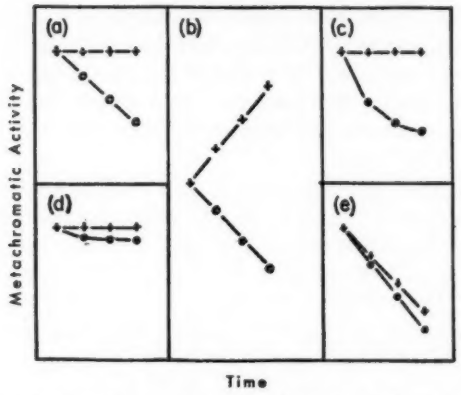


FIG. 4. Types of change in metachromatic values observed with heparin with various tissue extracts. Upper curve in each case represents values obtained with the control in which heparin and tissue extract were incubated separately for the time given, then mixed and precipitated immediately; lower curve represents values obtained after heparin and tissue extract were incubated together for the time shown. Time for samples—zero, one, two, three hours. Initial metachromatic value equivalent to 10.0–12.5 units of heparin.

setting up adequate controls, but this has not been the case. Typical results obtained with different tissue extracts during the course of the work are summarized in Fig. 4. The lower curve in each case is for the experimental series, i.e. the heparin assay value of the extracts obtained after heparin plus tissue extract was incubated at 37° C. for the times shown. The upper curves represent the controls, i.e. the values obtained when corresponding aliquots of heparin and enzyme have been incubated separately for the time shown, then combined and precipitated immediately with phenol. Fig. 4*a* shows a satisfactory reaction in which the control gives no change in value and the experimental values show a steady fall. However, many extracts show the phenomenon illustrated in Fig. 4*b*. If heparin and enzyme are mixed after they are incubated separately, the value is much greater than in the zero hour sample. Thus starting with 100 units of heparin, the assay after two hours' incubation reads 150 units. This is not due to a leaching out of heparin from the enzyme extract, because the enzyme after incubation still gives no metachromasia when precipitated by phenol without the addition of commercial heparin. If the activity of the enzyme were expressed as the difference between experimental and control values, then the value for the heparin destroyed after two hours would be several times that of the heparin added. This does not appear to be a reasonable measure of the enzyme activity, however, because of the failure to demonstrate release of heparin in the enzyme extract on incubation. We have therefore adopted the practice of expressing the activity as the decrease in the experimental value only. On this basis, *a*, *b*, *c*, of Fig. 4 represent active enzyme preparations, and *d* an inactive preparation. However, the control series can not be discarded because case *e* is found where both experimental and control values decrease. When

this occurs (as with the highest ammonium sulphate fractions of beef liver extracts), the decrease in activity in the experimental tubes is not considered to be due to enzyme activity. It probably represents the increased binding of heparin by albumins on denaturation. The increase in the control value in *b* resembles the phenomenon with urine described by Jaques, Napke, and Levy (7).

The reported method of purification gives a considerable concentration of enzyme. The final yield of Stage IV preparation is approximately 10% of the enzyme present in the unfractionated extract and represents about 250- to 600-fold purification of the enzyme with respect to protein nitrogen and specific activity. Presumably, this preparation is still crude. It should be noted that the method of preparation suggests that heparinase from ox liver is a globulin, as it is almost quantitatively precipitated by 30% saturated ammonium sulphate and is insoluble in distilled water. The rabbit liver heparinase, however, frequently gave a different result with ammonium sulphate, and was not always precipitated with this concentration of the salt. It seems that there may be a species difference in heparinase as there is a species difference in heparin as reported by Jaques (4) and Jaques, Waters, and Charles (8).

The observation that on precipitation of the liver extracts with alcohol and acetone, enzyme activity was found in the first and last fractions suggests that there may be either two enzymes with heparinase activity in liver extracts or that the enzyme is present in several different forms. It is probable that the final method of preparation using 33% saturation with ammonium sulphate includes only the first fraction. We have postponed investigating the second fraction. The association with the albumin fraction, which is responsible for non-enzymatic binding of heparin, increased the difficulty of investigating this fraction.

Because of the globulin nature of heparinase, it appeared that almost all the enzyme was precipitated by adjusting the dialyzate to pH 6 as described. However, for further purification a soluble form of heparinase was needed and this presented difficulties. Many attempts were made to dissolve this pH 6 precipitate without losing the enzyme activity. High and low pH dissolved the pH 6 precipitate, but inactivated the enzyme. Detergents of high molecular weight (e.g. Alconox) and surface active substances (the Triton series of Rohm and Haas CO.) were tested. These reagents dissolved the pH 6 precipitate, but inactivated the enzyme. Extraction of the pH 6 precipitate with water and saline in the cold (6° C.) and at room temperature (20° C.) were also tested without much success. The active enzyme was extracted only after the pH 6 precipitate was heated at 37° C. for one hour in saline at pH 7.0. This was rather difficult to explain solely on the basis of the enzyme being globulin. Finally, as with many enzymes that are associated with the globulin fraction, inactivation of the enzyme occurred very readily during the preparation processes. It was necessary to conduct all procedures below 10° C. and preferably at 5° C. or lower. On the basis of the many preparations

(active and inactive) made during the course of the work, it has been evident that different processes, including proteolytic enzymes, may be responsible for inactivation of the enzyme at various stages.

The optimum pH of 4.8 is close to the former results of Jaques (3). The inhibition of heparinase due to high concentration of heparin is an interesting observation and corresponds with the physiological findings of Jaques, Napke, and Levy (7) who concluded that after the metabolizing system of the animal body is saturated with heparin the latter is excreted in the form of the injected heparin instead of being metabolized. As pointed out by these authors, uroheparin does not appear to be the product of heparinase action, so we do not yet know the nature of the action of heparinase. Heparin is a marked inhibitor of several enzyme reactions (enzymes of blood coagulation, hyaluronidase, etc.) and it is rather interesting that heparin inhibits heparinase. Inhibition of heparin reactions by excess heparin was shown previously by Jaques, Bruce-Mitford, and Ricker (5) in the case of the metachromatic reaction with azure A and toluidine blue. Excess heparin causes suppression of this color reaction. It can be expected that the inhibition may cause some difficulty in the assay of the enzyme in tissue extracts, etc. The possible presence, in the extract, of heparin from the tissue or the presence of only small amounts of enzyme may mean that the enzyme activity is suppressed by excess heparin and thus reported as negative. While the kinetics of the inhibition of heparinase by heparin require a more extensive investigation, the results reported in Fig. 2*c* suggest that the lower the enzyme concentration, the lower the concentration of heparin required for inhibition and therefore the lower the optimum substrate concentration for the enzyme.

Acknowledgment

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NOTE

GLUCOSURIA IN RATS RECEIVING BRITISH ANTI-LEWISITE (BAL)[1]

By Beverley Tamboline, A. T. Matheson,[2] and S. H. Zbarsky

In 1947 Spray (7) reported that injection of BAL into rats led to an increased urinary excretion of glucuronic acid during the subsequent 24 hr. As part of a study on the metabolic products excreted in the urine of rats given BAL, we have also examined the urine for a possible increased glucuronic acid content, but have been unable to detect any such increase. We have observed, however, a marked glucosuria during the first few hours following the injection of BAL. The details of these studies are reported below.

The experiments were carried out on male rats of the Wistar strain, weighing approximately 200 gm. each. In a typical study, urine was collected daily for four or five days from a group of three rats. Each animal was then injected intraperitoneally with 100 mgm./kgm. body weight of BAL dissolved in 0.5 ml. of propylene glycol, and urine was collected from the group for the periods 0–3, 3–6, 6–12, 12–24, and 24–48 hr. after the injection. To test for the presence of glucuronic acid, filter paper chromatograms of each collection of urine were prepared using as a solvent *tert*-butanol–water (75 : 25 by volume) (8). After the chromatograms had been dried in air, they were sprayed with the benzidine reagent described by Miller and Kraemer (5), and used by them to indicate the presence of reducing substances, including carbohydrates. On the chromatograms of the 0–3 and 3–6 hr. urines, a prominent brown spot appeared with the same R_f value as that given by both glucuronic acid and glucose, which are not separated by the *tert*-butanol–water solvent. In order to determine the nature of the material in the urine, a second series of chromatograms was prepared using the *n*-butanol – acetic acid – water solvent described by Partridge (6). With this solvent, which gives satisfactory chromatographic separation of glucose and glucuronic acid, the 0–3 and 3–6 hr. urine chromatograms showed prominent spots with R_f values corresponding to that of glucose (R_f = 0.18). No glucose spots were observed with preinjection urines or with those collected more than six hours after injection of BAL. The 0–3 and 3–6 hr. urines also gave strongly positive tests with Benedict's solution.

On all the chromatograms prepared with Partridge's solvent (6) only faint spots of glucuronic acid (R_f = 0.33) were observed and no evidence of an increased excretion of glucuronic acid after BAL injection was detected. The possibility existed, however, that there was an increased excretion of glucuronic acid in the form of a glucuronide. The latter would not react with the benzidine spray to give a colored spot on the paper chromatograms. In order to release any bound glucuronic acid a sample of postinjection urine was

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adjusted to pH 4.5 and incubated with calf spleen β-glucuronidase for eight hours at 37° C. Chromatograms of this urine gave no indication of an increase in free glucuronic acid.

Similar experiments were carried out with two commercial preparations of BAL.[3] It was of interest to note that when the rats were given 25 mgm./kgm. body weight of BAL the urines reacted negatively with Benedict's solution, whereas strongly positive tests, corresponding to a glucose concentration of 2%, were obtained with urine excreted during the first six hours by animals receiving 75 mgm./kgm. body weight of BAL. Tests for the presence of ketoses gave negative results. Further examination of these urines by paper chromatography, this time using the solvent described by Gustaffson, Sundman, and Lindh (3), also demonstrated that the carbohydrate was glucose. By comparing the areas of the glucose spots with those obtained from solutions of known glucose content, a urinary glucose concentration of 1–2% was indicated. Further evidence that the reducing sugar in the urine was glucose was obtained by polarimetric examination (4). The urine samples were dextrorotatory, a typical sample having a specific rotation of $[\alpha]_D^{22} = +50.5$, assuming the sugar to be glucose. The optical activity was destroyed by treatment of the urine with yeast.

We also examined the urine of a human subject being treated with BAL for hepatolenticular degeneration[4] but found no evidence of glucosuria. It was felt that the dose of BAL being administered was below that required to produce glucosuria.

The finding of a temporary marked glucosuria in rats injected with BAL is indicative of a rapid interference with normal carbohydrate metabolism. In this connection, Durlacher *et al.* (2) reported that in dogs given lethal doses of BAL there is a loss of liver glycogen and a temporary hyperglycemia. The work of Barron, Miller, and Meyer (1), who found that incubation with BAL *in vitro* destroyed the hypoglycemic effect of insulin in rabbits, suggests that the effect of BAL may be to inactivate insulin. If an inactivation of insulin occurs *in vivo*, glucosuria would be an expected result.

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[3]*Purchased from Bios Laboratories, Inc., New York 23, N.Y., and Hynson, Westcott and Dunning, Inc., Baltimore 1, Md.*

[4]*The urine of this patient was made available to the authors through the courtesy of Dr. W. Fister and Mr. R. Baker of the Crease Clinic, Essondale, B.C.*

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